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ANTAGONISM OF APOMORPHINE-INDUCED PECKING IN PIGEONS

BY

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(*Received June* 24, 1960)

Central nervous system stimulants, tranquillizers and other central nervous system depressants, antiemetics, antihistamine drugs and autonomic blocking agents were examined for their ability to prevent the pecking response in pigeons induced by apomorphine (250 μg/kg intramuscularly). Reduction in the proportion of positive responses or significant increase in the latent period of pecking were taken as the criterion of effectiveness. Protection was afforded by caffeine, lysergic acid diethylamide, morphine, rauwolscine, triflupromazine and yohimbine. In addition, a significant increase in latent period was produced by artane, pentobarbitone, benactyzine, 2-bromolysergic acid diethylamide, cyclizine, diphenhydramine, ergotoxine, hyoscine, promethazine, 5-(2-chloroethyl)-4-methylthiazole and trimethobenzamide. Most of these drugs influenced the pecking and emetic responses to apomorphine in an identical manner. It is possible that identical receptors may be concerned with apomorphine pecking (in pigeons) and emesis (in other species).

Dhawan & Saxena (1960a, b) showed that apomorphine makes pigeons peck. The latent period, intensity and duration of the response were related to the dose. Neither conditioning nor tolerance was observed on chronic administration. The mechanism of this phenomenon is not known. In the present investigation, various groups of neuropharmacological agents have been screened for their ability to prevent pecking caused by apomorphine in order to find out the probable mechanism of this phenomenon. The drugs tested include central nervous system stimulants, tranquillizers and other central nervous system depressants, antiemetics, antihistamine drugs and antagonists of the autonomic nervous system.

A preliminary report of part of this work was presented before the 1959 Session of the Association of Physiologists and Pharmacologists of India at Poona (Dhawan & Saxena, 1960a).

METHODS

The methods used have been described in a previous paper (Dhawan & Saxena, 1960b). Two hundred and ninety pigeons (weight 200 to 400 g) of either sex were used. The birds were not used more than twice a week. During the period of observation birds were placed separately in small cages in a quiet room and food and water were withdrawn.

Pecking was induced by intramuscular injection of 250 μg/kg (approximately $4 \times$ ED50—Dhawan & Saxena, 1960b) of apomorphine hydrochloride (British Drug Houses). The time elapsing between the drug administration and the injection of apomorphine has been shown with each drug. (See Table 1.) In most cases no data were available regarding the time of

TABLE 1

EFFECT OF DRUGS ON APOMORPHINE-INDUCED PECKING IN PIGEONS

Drugs were given intramuscularly except wherein otherwise indicated. Values of latent period (L.P.) marked with an asterisk (*) differ significantly ($P=0.05$) from the control group values

Drug	No. of birds	Dose (mg/kg)	Apomorphine challenge after	% Positive responses to apomorphine	Mean L.P. in min±s.e.	PD50 in mg/kg±s.e. ($P=0.05$)
No antagonist (apomorphine alone)	60	0		100	4·3±0·2	
CNS stimulants:						
Caffeine citrate	10	6·25	30 min	100	5·2±0·8	
	20	9·0		80	6·6±1·5	16·27±4·98
	10	12·5		60	8·0±1·2*	
	10	25·0		30	9·6±0·1*	
	10	100·0		0	—	
Cocaine hydrochloride	10	3·0	30 min	90	5·3±0·4	
	10	6·0		50	7·2±1·1	6·59±4·2
	10	12·0		10	11·0±0·0*	
	10	24·0		20	10·5±0·5*	
Procaine hydrochloride	10	20·0	30 min	90	6·0±1·5	
5-Hydroxytryptamine	10	0·25	30 min	90	4·8±0·7	
	10	0·5		80	4·2±0·8	
	10	1·0		80	6·6±1·2	
	10	2·0		100	5·9±1·1	
Iproniazid phosphate	10	150·0	2 hr	90	4·3±0·37	
Lysergic acid diethylamide	10	0·025	15 min	70	8·1±2·7	
	10	0·05		60	8·8±2·9	0·052±0·031
	10	0·1		20	5·5±1·6	
	10	0·2		20	5·5±0·7	
2-Bromolysergic acid diethylamide	10	0·2	15 min	70	9·4±1·2*	
	10	0·4		90	7·5±1·0*	
Methylamphetamine hydrochloride	10	2·5	30 min	90	5·3±1·1	
	10	5·0		80	6·6±2·8	
Methylphenidate hydrochloride (oral)	10	5·0	30 min	100	4·5±0·5	
Metrazol	10	25·0	30 min	90	6·0±1·2	
Strychnine sulphate	10	0·5	30 min	100	5·8±0·7	
Tranquillizers:						
Azacyclonol hydrochloride (oral)	10	20·0	30 min	100	6·7±0·05	
	10	50·0		80	4·0±0·4	
	10	100·0		100	5·6±0·7	
Benactyzine hydrochloride	10	3·0	30 min	90	13·3±1·9*	
	10	6·0		70	8·5±2·0	
Chlorpromazine hydrochloride	10	1·0	30 min	80	6·6±1·1	
	10	5·0		50	8·2±1·1*	4·44±2·56
	10	10·0		20	7·0±0·0*	
	10	25·0		20	4·5±1·5	
Hydroxyzine hydrochloride	10	10·0	30 min	100	3·2±0·54*	
Meprobamate (oral)	10	10·0	30 min	100	3·2±0·17*	
Reserpine phosphate	10	0·5	30 min	100	3·6±0·21*	
	10	2·5		100	5·7±0·9*	
Other CNS depressants:						
Diphenylhydantoin sodium (oral)	10	40·0	2 hr	100	5·5±1·1	
Levorphanol tartrate	10	2·0	30 min	90		

Hydroxyzine hydrochloride	10	10·0	30 min	100	3·2±0·17*	
Meprobamate (oral)	10	10·0	30 min	100	3·6±0·21*	
Reserpine phosphate	10	0·5	30 min	100	5·7±0·9*	
	10	2·5				
Other CNS depressants:						
Diphenylhydantoin sodium (oral)	10	40·0	2 hr	100	5·5±1·1	
Levorphanol tartrate	10	2·0	30 min	90	7·8±1·3*	
Mephenesin	10	50·0	30 min	90	4·1±0·4	
Methylpentynol carbamate (oral)	10	25·0	30 min	90	3·1±0·2	
Morphine tartrate	10	1·0	30 min	90	5·5±0·8	
	10	5·0		60	7·7±1·4	
	10	10·0		60	10·3±2·3*	17·24±0·17
	10	20·0		60	10·0±0·9	
	10	40·0		30	8·0±1·8	
Nalorphine hydrochloride	10	10·0	30 min	90	7·0±0·5*	
	10	15·0		90	3·2±0·3*	
Phenobarbitone sodium	10	25·0	30 min	90	3·8±0·5	
5-(2-Chloroethyl)-4-methylthiazole	10	40·0	30 min	90	9·9±2·0*	
Trimethadione	10	200·0	1 hr	100	4·2±0·5	
Antiemetics:						
Promethazine theoclate (oral)	10	25·0	30 min	90	3·8±0·5	
Cyclizine hydrochloride (oral)	10	50·0	3 hr	100	2·5±0·59	
		100·0		100	9·9±1·5*	
Hyoscine hydrobromide	10	2·0	30 min	80	11·6±3·1*	
	10	4·0		90	7·6±1·8	
Prochlorperazine hydrochloride	10	0·38	30 min	60	8·3±1·9	
	10	0·75		30	9·0±2·2*	0·45±0·35
	10	1·5		20	9·0±3·0	
	10	3·0		10	32·0±0·0*	
Trimethobenzamide hydrochloride	10	20·0	30 min	80	8·0±0·3*	
	10	40·0		90	8·0±1·4	
Trifluprομazine hydrochloride	10	0·25	30 min	80	7·0±1·7	
	10	0·50		40	8·0±1·2*	0·51±0·3
	10	1·0		30	14·3±3·8*	
Antihistaminics:						
Diphenhydramine hydrochloride (oral)	10	25·0	30 min	90	7·0±1·9	
Promethazine hydrochloride	10	25·0	30 min	100	10·5±1·3*	
Triprolidine hydrochloride (oral)	10	5·0	30 min	100	4·9±2·1	
Autonomic blocking agents:						
Benzhexol hydrochloride	10	2·0	30 min	80	5·25±1·0	
	10	6·0		70	13·8±2·6*	
Atropine sulphate	10	3·0	30 min	90	5·5±1·2	
Hyoscine butylbromide	10	10·0	30 min	100	6·6±1·2	
Dihydroergotamine methanesulphonate	10	0·5	30 min	100	4·9±0·6	
Ergotoxine ethanesulphonate	10	0·5	30 min	100	4·9±1·7	
Rauwolscine hydrochloride	10	0·3	30 min	100	5·2±0·6	
	10	0·4		80	4·0±0·7	0·51±0·11
	10	0·5		50	6·0±2·0	
	10	0·6		10	10·0±0·0*	
Yohimbine hydrochloride	10	0·15	30 min	90	4·5±1·2	
	10	0·30		50	6·5±1·2	
	10	0·6		20	5·5±2·6	0·32±0·11
	10	5·0		0	—	

peak action of a particular drug in pigeons. Hence the time at which the peak of other actions (mostly observed in other species) has been reported in literature was used in the present investigation. It is, however, possible that in certain cases apomorphine challenge may not have coincided with the peak effect of the drug. Similarly, the dose levels of various drugs tested were chosen on the basis of doses employed by various workers for other effects in pigeons or in other species (if data for pigeons were not available). The birds were observed for at least 1 hr after apomorphine injection. Only birds pecking more than 10 times during this period (Dhawan & Saxena, 1960b) were considered to be responding to the apomorphine challenge. In these cases the latent period was the period elapsing between apomorphine administration and the first act of pecking. In addition birds were also observed for any signs of neurotoxicity, such as excitement, sedation, or ataxia.

Drugs to be tested were administered intramuscularly (aqueous solution) in the pectoral muscles or orally by allowing a suspension (in 4% homogenized gum acacia solution) to drop in the mouth from a tuberculin syringe. Each dose was tested in one batch of ten pigeons. Since it was not feasible to determine accurately the duration of pecking or to assess its intensity quantitatively, the effect of a drug was assessed on the basis of changes in the latent period of pecking and in % positive responses to apomorphine. If a drug gave protection, graded doses were given and at least three points were obtained between levels of 100% protection and no protection. Regression lines were then obtained by the method of Bliss (1935a, b, c). The lines were tested for goodness of fit by the χ^2 test as described by Finney (1952) and PD50 (dose protecting 50% pigeons from the effect of apomorphine) values along with their standard errors ($P=0.05$) calculated. The significance of drug-induced alteration in latent period was decided by the "Standard Error of the Mean" method of Tippet (1925), at 95% confidence level ($P=0.05$).

RESULTS

Six groups of 10 pigeons each received apomorphine (250 μg/kg) alone. These groups were tested between the various groups treated with additional drugs and a 100% pecking response was obtained with a mean latent period of 4.3 ± 0.2 (s.e.) min.

Central nervous system stimulants

Eight stimulants, caffeine citrate, cocaine hydrochloride, iproniazid phosphate, lysergic acid diethylamide, methylamphetamine hydrochloride, methylphenidate hydrochloride, metrazol and strychnine sulphate, were studied. In addition 5-hydroxytryptamine creatinine sulphate (serotonin) and 2-bromolysergic acid diethylamide were also included because of their possible significance in activity of the brain, and procaine hydrochloride for comparison with cocaine. The results have been summarized in Table 1. Protection was afforded by caffeine, cocaine and lysergic acid diethylamide. Procaine was ineffective (Table 1). Three drugs, 2-bromolysergic acid diethylamide, caffeine and cocaine, significantly prolonged the latent period (Table 1). The pigeons treated with 0.1 and 0.2 mg/kg lysergic acid diethylamide were sedated. Similar observations with this drug have also been reported by Hoffman (1958). Pigeons receiving 5 mg/kg methylamphetamine were very much excited, as evidenced by excessive movements inside the cage, fluttering of feathers and increased head movements. Larger doses, therefore, could not be used. Similarly 20% of the pigeons treated with 2 mg/kg serotonin died, precluding the testing of larger doses.

Tranquillizers

Six drugs, azacyclonol hydrochloride, benactyzine hydrochloride, chlorpromazine hydrochloride, hydroxyzine hydrochloride, meprobamate and reserpine phosphate, were tested. The results are shown in Table 1. Protection was afforded by chlorpromazine. Some protection was afforded by benactyzine in doses tested. Larger doses of benactyzine could not be tested because pigeons appeared confused and excited even with the 6 mg/kg dose. Thus many pigeons tried to escape out of the cage even though the cage was closed. This behaviour was not seen with other agents even though the pigeons were excited, and was probably an indication of the confusion produced by benactyzine. A significant decrease in latent period was produced by reserpine, hydroxyzine and meprobamate; chlorpromazine and benactyzine significantly increased it, while azacyclonol was without effect. Birds treated with larger doses of chlorpromazine and azacyclonol were unduly sedated. Occasional pecking (never more than 10 times during the observation period) was seen in some of the birds treated with benactyzine.

Other central nervous system depressants

Hypnotics. Methylpentynol carbamate, phenobarbitone sodium, pentobarbitone sodium and methyl-4β-chlorethyl-5 thiazol (Charonnat, Lechat & Chareton, 1953) were screened. Some protection was afforded by pentobarbitone (10 mg/kg), methylpentynol and methyl-4β-chlorethyl-5 thiazol at neurotoxic dose levels (Table 1). Phenobarbitone failed to protect even at neurotoxic dose levels (25 mg/kg, Table 1). Ataxia was the main neurotoxic manifestation in all the cases. The pigeons were unable to maintain the posture and frequently fell on their sides. Further, they staggered while moving inside the cage. Latent period was significantly increased by neurotoxic (ataxic) doses of all the agents except methylpentynol, which decreased it. While the increase in latent period may be due to ataxia produced by these drugs, it is difficult to explain the decrease produced by methylpentynol.

Analgesics. Morphine tartrate, levorphanol tartrate and nalorphine hydrochloride were tested. Protection was afforded by morphine (PD50: 17.24 ± 0.17 mg/kg). The pigeons were very much sedated with 2.0 mg/kg levorphanol, thereby preventing the testing of larger doses. All the agents significantly increased the latent period for pecking (Table 1).

Two anticonvulsants (diphenylhydantoin sodium and trimethadione) and one internuncial blocking agent (mephenesin) were also included in the study, but none of them gave any protection.

Antiemetic drugs. Apomorphine-induced pecking in pigeons has some resemblance with apomorphine-induced vomiting in other species. For example, both have a short latent period and both can be produced by minute amounts of apomorphine. To investigate this similarity further, a number of antiemetic agents were tested for their ability to antagonize this pecking. The drugs tested were cyclizine hydrochloride, hyoscine hydrobromide, prochlorperazine hydrochloride, promethazine theoclate (Avomine), trimethobenzamide hydrochloride (Tigan) (Schallek, Heise, Keith & Bagdon, 1959) and trifluprazine hydrochloride. In addition chlorpromazine (considered with tranquillizers) is also an antiemetic agent.

Protection was afforded by chlorpromazine (*vide supra*), prochlorperazine (PD50: 0.65 ± 0.34 mg/kg) and triflupromazine (0.7 ± 0.3 mg/kg). Koster (1957) reported a reduction in the intensity of pecking by 75 mg/kg cyclizine orally. However, no appreciable alteration in intensity was seen in the present investigation even after 100 mg/kg dose. Birds receiving 4.0 mg/kg hyoscine were very much excited.

Antihistamine drugs

Many antihistamine agents antagonize apomorphine vomiting in animals (Boyd, Cassell, Boyd & Miller, 1955). As some antiemetic agents could block apomorphine-induced pecking some antihistamines were tested. Diphenhydramine hydrochloride, promethazine hydrochloride and triprolidine hydrochloride (Actidil) were tested. None of the agents afforded significant protection (Table 1). The latent period was increased by promethazine (cf. effect of other phenothiazines—*vide supra*).

Antagonists of the autonomic nervous system

Antiadrenergic drugs. Ergotoxine (Hatcher & Weiss, 1923) and dibenamine (Freedman & Giarman, 1956) are known to antagonize vomiting induced by apomorphine. Hence 4 antiadrenergic drugs, dihydroergotamine methanesulphonate, ergotoxine ethanesulphonate, rauwolscine hydrochloride and yohimbine hydrochloride, were studied. Protection was afforded by rauwolscine ($PD50 = 0.51 \pm 0.01$) and yohimbine ($PD50 = 0.32 \pm 0.11$). Ergotoxine produced a significant increase in the latent period (see Table 1) only.

Anticholinergic drugs. Bernheim & Bernheim (1936) and Kuhn & Surles (1938) have shown that apomorphine has a high anticholinesterase activity. To investigate a possible cholinergic mechanism of pecking 5 cholinergic antagonists, atropine sulphate, benactyzine hydrochloride, hyoscine butylbromide (Buscopan), hyoscine hydrobromide and benzhexol hydrochloride (Artane), were tested. Two of these agents have already been mentioned, benactyzine as a tranquillizer and hyoscine as an antiemetic. None of the agents tested afforded protection. Latent period was significantly prolonged by artane, benactyzine and hyoscine.

DISCUSSION

It is difficult to localize the site or mechanism of this effect of apomorphine in pigeons on the basis of the present results. It might be some sort of feeding hallucination (Koster, 1957), but, as pointed out in a previous communication (Dhawan & Saxena, 1960b), it is not produced by other hallucinogenic agents. Again, it cannot be blocked by azacyclonol—believed to be a specific antihallucinogenic drug (Brown, Feldman & Braun, 1955)—in the doses tested (see tranquillizers).

The results show that protection is afforded by caffeine, chlorpromazine, cocaine, lysergic acid diethylamide, morphine, rauwolscine, trifluprom azine and yohimbine. It is interesting to note that 2-bromolysergic acid diethylamide, which does not share the central action of lysergic acid diethylamide (Rothlin, 1957), is ineffective. Similarly, it fails to block apomorphine-induced emesis in dogs (Dhawan & Gupta, 1960b) which is antagonized by lysergic acid diethylamide (Dhawan & Gupta, 1960a). It is not possible to correlate the anti-pecking effect

TABLE 2

CORRELATION BETWEEN EFFECT OF DRUGS ON APOMORPHINE-INDUCED PECKING AND EMESIS

+ indicates blockade, − no blockade, ± only latent period prolonged (pecking) or inconclusive results (emesis), and ? effect not studied so far.

Drug	Blockade of pecking	Blockade of emesis	Reported by
Chlorpromazine	+	+	Brand, Harris, Borison & Goodman, 1954
Lysergic acid diethylamide	+	+	Dhawan & Gupta, 1960a
Morphine	+	+	Hatcher, 1924
Prochorperazine	+	+	Boyd & Cassell, 1957
Triflupromazine	+	+	Piala, High, Hassert, Burke & Craver, 1959
Cocaine	+	?	
Caffeine	+	?	
Rauwolscine	+	?	
Yohimbine	+	?	
Atropine	−	−	Eggles ton, 1916
Azacyclonol	−	−	Boyd& Cassell, 1957
2-Bromolysergic acid diethylamide	±	−	Dhawan & Gupta, 1960b
Hyoscine	±	−	Boyd and Cassell, 1957
Cyclizine	±	+	Koster, 1957
Diphenhydramine	±	+	Boyd, Cassell, Boyd & Miller, 1955
Ergotoxine	±	+	Hatcher & Weiss, 1923
Methylamphetamine	−	+	Boyd & Cassell, 1957
Promethazine	±	+	Boyd & Cassell, 1957
Reserpine	−	±	Malhotra & Sidhu, 1956 Ballinger & Borison, 1957
Trimethobenzamide	±	+	Schallek, Heise, Keith & Bagdon, 1959

TABLE 3

LACK OF CORRELATION BETWEEN EFFECTS OF DRUGS ON LATENT PERIOD FOR PECKING AND PROTECTION AFFORDED BY THEM

+ indicates significant increase of latent period/protection ; − a significant decrease in latent period ; and 0 no effect.

Agent	Effect on latent period	Protection
Benzhexol	+	0
Benactyzine	+	0
Caffeine	+	+
Chlorpromazine	+	+
Cocaine	+	+
Cyclizine	+	0
Ergotoxine	+	0
Hydroxyzine	−	0
Hyoscine	+	0
Levorphanol	+	0
Lysergic acid diethylamide	0	+
2-Bromolysergic acid diethylamide	+	0
Meprobamate	−	0
Morphine	+	+
Phenobarbitone	+	0
Prochlorperazine	+	+
Promethazine	+	0
Rauwolscine	+	+
Reserpine	−	0
Triflupromazine	+	+
Yohimbine	0	+

of the various drugs with their central nervous system stimulant, tranquillizing, central nervous system depressant, antihistamine or autonomic blocking activity.

However, many agents have influenced the pecking and emetic response to apomorphine in an identical manner (see Table 2). It has not been possible to antagonize apomorphine-induced pecking with a drug that is known to be ineffective against apomorphine-induced emesis. With some of the drugs found effective against pecking, no reports as regards their activity against apomorphine emesis are available (see group 2 in Table 2). These drugs are being tested against apomorphine emesis and the results will be reported later. It is possible that the cellular receptors involved in the mechanism of apomorphine-induced pecking in pigeons may be similar to that of apomorphine-induced emesis in other species.

It has been pointed out earlier (see methods) that two criteria—effect on the latent period and changes in % positive responses—were employed for assessing the drug effects. In nearly all cases (except with lysergic acid diethylamide and yohimbine) drugs affording protection also prolonged the latent period. On the contrary there were many agents which produced an increase in latent period without affording protection even in higher doses (see Table 3).

The authors are grateful to the following for supplying the drugs mentioned against their names: Burroughs Wellcome, Bombay (cyclizine, methylamphetamine, and triprolidine); Ciba Pharma, Bombay (reserpine and methylphenidate); Laboratories Du Dr. Debat, Paris (5-(2-chloroethyl)-4-methylthiazole); Hoffman La Roche Inc., Basel (iproniazid and tigan); Dr. J. D. Kohli, Central Drug Research Institute, Lucknow (rauwolscine); May & Baker, Delhi (prochlorperazine); Merrell—National Labs., New York (azacyclonol); Chas. Pfizer & Co., New York (hydroxyzine); Sandoz (Private), Bombay (2-bromolysergic acid diethylamide, 5-hydroxytryptamine, and lysergic acid diethylamide); and Sarabhai Chemicals, Baroda (trifluprom azine).

REFERENCES

BALLINGER, C. M. & BORISON, H. L. (1957). Comparison of Cyclizine (Marezine) with chlorpromazine, promethazine and reserpine against drug-induced emesis in dogs. *J. Pharmacol. exp. Ther.*, **119**, 131.

BERNHEIM, F. & BERNHEIM, M. L. C. (1936). Action of drugs on the choline esterase of the brain. *J. Pharmacol. exp. Ther.*, **57**, 427–436.

BLISS, C. I. (1935a). The calculation of the dosage-mortality curve. *Ann. appl. Biol.*, **22**, 134–167.

BLISS, C. I. (1935b). The comparison of dosage-mortality rate. *Ann. appl. Biol.*, **22**, 307–333.

BLISS, C. I. (1935c). Estimating the dosage-mortality curve. *J. Econ. Ent.*, **28**, 646–647.

BOYD, E. M. & CASSELL, W. A. (1957). Agents affecting apomorphine-induced vomiting. *J. Pharmacol. exp. Ther.*, **119**, 390–393.

BOYD, E. M., CASSELL, W. A., BOYD, C. E. & MILLER, J. K. (1955). Inhibition of the apomorphine-induced vomiting syndrome by antihistaminic drugs. *J. Pharmacol. exp. Ther.*, **115**, 299–309.

BRAND, E. D., HARRIS, T. D., BORISON, H. L. & GOODMAN, L. S. (1954). The anti-emetic activity of 10-(γ-dimethylaminopropyl)-2 chlorophenothiazine (chlorpromazine) in dog and cat. *J. Pharmacol. exp. Ther.*, **110**, 86–92.

BROWN, B. B., FELDMAN, R. & BRAUN, D. L. (1955). Pharmacological studies of an LSD antagonist, 4-piperidyl, diphenyl carbinol hydrochloride. *Fed. Proc.*, **14**, 322.

CHARONNAT, R., LECHAT, P. & CHARETON, J. (1953). *Ann. Pharm. Franc.*, **11**, 17, 26 and 729, quoted from FOLIN, S., BALIER, C., AZOULAY, J., GUEN, C. L. and ROBERT, M. (1958). Originalité d'action du S.C.T.Z. en therapeutique psychiatrique. *Ann. Med. Psychol.*, **1**, 1–8.

DHAWAN, B. N. & GUPTA, G. P. (1960a). Some further observations on LSD-25 morphine antagonism. *Ind. J. Physiol. Pharmacol.*, **4**, 116.

DHAWAN, B. N. & GUPTA, G. P. (1960b). Anti-emetic activity of D-lysergic acid diethylamide. *J. Pharmacol. exp. Ther.* In the press.

DHAWAN, B. N. & SAXENA, P. N. (1960a). Observations on apomorphine-induced pecking in pigeons. *Ind. J. Physiol. Pharmacol.*, **4**, 115.

DHAWAN, B. N. & SAXENA, P. N. (1960b). Apomorphine-induced pecking in pigeons. *Brit. J. Pharmacol.*, **15**, 285–289.

EGGLESTON, C. (1916). The antagonism between atropine and certain central emetics. *J. Pharmacol. exp. Ther.*, **9**, 11–25.

FINNEY, D. J. (1952). *Probit Analysis*, 2nd ed., p. 24. Cambridge : University Press.

FREEDMAN, D. X. & GIARMAN, N. J. (1956). Apomorphine test for tranquillizing drugs: effect of dibenamine. *Science*, **124**, 264.

HATCHER, R. A. (1924). Mechanism of vomiting. *Physiol. Rev.*, **4**, 479–504.

HATCHER, R. A. & WEISS, S. (1923). Studies on vomiting. *J. Pharmacol. exp. Ther.*, **22**, 139–193.

HOFFMAN, R. A. (1958). Effect of LSD-25 on body temperature of pigeons. *Amer. J. Physiol.*, **195**, 747–758.

KOSTER, R. (1957). Comparative studies of emesis in pigeons and dogs. *J. Pharmacol. exp. Ther.*, **119**, 406–417.

KUHN, H. H. & SURLES, D. (1938). The action of various members of the morphine series and emetine upon the choline esterase of the brain. *Arch. int. Pharmacodyn.*, **58**, 88–92.

MALHOTRA, C. L. & SIDHU, R. K. (1956). The anti-emetic activity of alkaloids of Rauwolfia serpentina. *J. Pharmacol. exp. Ther.*, **116**, 123–129.

PIALA, J. S., HIGH, J. P., HASSERT, Jr., G. L., BURKE, J. C. & CRAVER, B. N. (1959). Pharmacological and acute toxicological comparisons of trifluprομazine and chlorpromazine. *J. Pharmacol. exp. Ther.*, **127**, 55–65.

ROTHLIN, E. (1957). Pharmacology of lysergic acid diethylamide and some of its related compounds. *J. Pharm. Pharmacol.*, **9**, 569–587.

SCHALLEK, W., HEISE, G. A., KEITH, E. F. & BAGDON, R. E. (1959). Anti-emetic activity of 4-(2-dimethylaminoethoxy)-N-(3,4,5-trimethoxybenzoyl) benzylamine hydrochloride. *J. Pharmacol. exp. Ther.*, **126**, 270–277.

TIPPET, L. H. C. (1925). *Biometrica*, **17**, 364. Quoted from R. A. HOFFMAN above.

Brit. J. Pharmacol. (1961), **16**, 146–152.

ANTIHISTAMINASE ACTIVITY OF SERPENTINE

BY

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Serpentine, an alkaloid of Rauwolfia serpentina, specifically potentiates histamine responses of guinea-pig ileum, uterus, and tracheal chain. It also inhibits histaminase *in vitro*. Its antihistaminase activity is equivalent to that of aminoguanidine.

Chatterjee & Hausler (1955) have reported antiacetylcholine and antihistamine actions of a total alkaloidal preparation of Rauwolfia serpentina on the guinea-pig ileum.

During a study of the pharmacology of serpentine, one of the alkaloids of Rauwolfia serpentina, by one of the authors (Sachdev, 1958), it was noted that serpentine markedly and consistently potentiated the response of the guinea-pig ileum to histamine. Arunlakshana, Mongar, and Schild (1954) demonstrated that potentiation of the histamine response of guinea-pig ileum, uterus, and tracheal chain by semicarbazide, hydroxylamine, guanidine, and aminoguanidine could be attributed to inhibition of diamine oxidase or histaminase. Murexine and dihydromurexine (Tabachnick & Roth, 1957) and stilbamidine (Lindell & Westling, 1954) have also been reported to show similar potentiation of histamine responses and inhibition of histaminase. Sachdev & Aiman (1957), in a preliminary report, showed that serpentine potentiated the action of histamine on the guinea-pig ileum and inhibited histaminase. In this paper we have demonstrated a potentiation of histamine response of organs shown by Arunlakshana, Mongar & Schild (1954) to be rich in histaminase and inhibition of histaminase *in vitro* by serpentine, and have compared its antihistaminase activity with that of aminoguanidine.

METHODS

Guinea-pig ileum. The terminal part of the guinea-pig ileum was obtained after killing the animal with a blow on its head. About an inch of this was cleaned and suspended in a 10-ml. isolated organ bath containing oxygenated Tyrode solution at 36 to 37° C. Repeated responses to histamine (histamine acid phosphate, British Drug Houses) were elicited every 2 min, until the preparation became stabilized; the drug was allowed to act for 30 sec. A few graded responses of histamine were elicited. A suitable dose was chosen and repeated 4 to 5 times. This dose of histamine (expressed as the base) was usually between 0.01 and 0.03 μg/ml. Thirty sec before each dose one of the potentiating drugs (serpentine and aminoguanidine) was added to produce one of the following concentrations in the bath: 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} g/ml. The potentiating drug was added until maximum potentiation was obtained. Thereafter it was omitted and repeated responses of histamine elicited until these

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returned to control levels. The potentiation was measured in terms of an equivalent dose of histamine. The procedure of Arunlakshana, Mongar & Schild (1954) was followed. In some experiments atropinized ileum was used.

Guinea-pig uterus. This preparation was suspended in the same way as ileum. The responses to histamine were elicited every 5 min. Histamine was allowed to act for 1 min and then washed out. The potentiating drug was added in the same concentration as used for the ileum but for 1 min before the response of histamine was elicited.

Guinea-pig tracheal chain. The method described by Castillo & de Beer (1947) was followed. The rings of the trachea were, however, cut in the middle of the cartilage and tied end to end.

The histamine responses were registered for 10 min. After eliciting responses to a few graded doses of histamine usually between 10^{-7} and 5×10^{-6} g/ml., one of the previously mentioned concentrations of the potentiating drug was added 4 min before the next histamine response. Histamine was added to the bath without removing the potentiating drug.

Histaminase in vitro. 5 mg/ml. of histaminase (Torantil, Hoechst) solution in phosphate buffer at *p*H 7.2 was incubated for 30 min at 37° C in an atmosphere of oxygen in a metabolic shaker, with 10^{-6} g/ml. of histamine as substrate, with and without various concentrations of serpentine (10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, and 10^{-3} g/ml.). After incubation the histaminase was destroyed by boiling. These solutions were assayed for histamine on atropinized guinea-pig ileum. At least three assays were done for each solution. As histaminase is inactive in the absence of oxygen, one incubation was carried out anaerobically as a control. The final solution after heat inactivation of the histaminase was assayed for histamine. Serpentine was used as the hydrochloride. The doses mentioned in the text represent the quantity of the base.

RESULTS

Guinea-pig ileum. Serpentine in a concentration of 10^{-9} g/ml. produced minimal potentiation. Maximum potentiation (doubling the responses to histamine) was seen with 10^{-8} and 10^{-7} g/ml. of serpentine (Fig. 1). Higher concentrations than

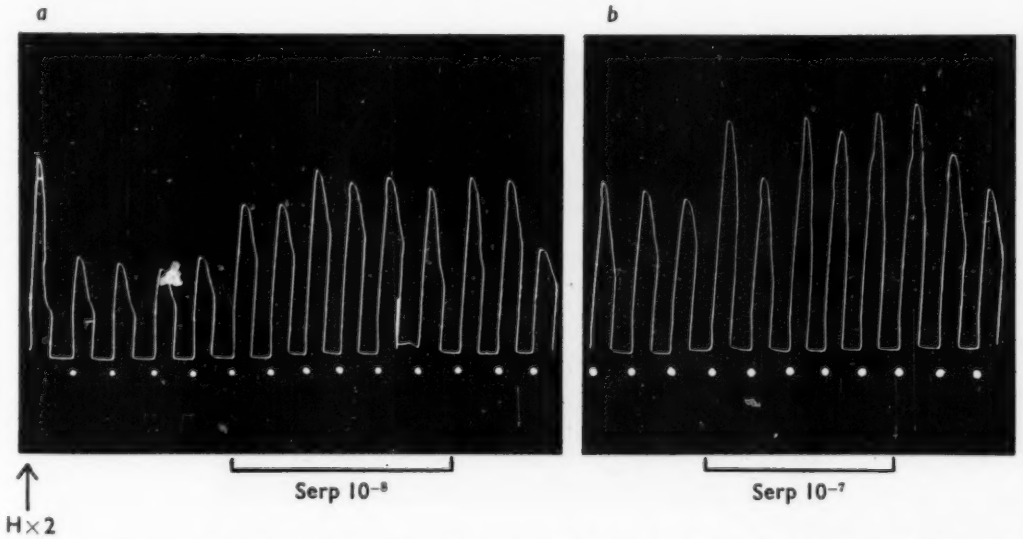

Fig. 1. Responses of guinea-pig ileum to 0.02 μg/ml. of histamine, added at white dots, and washed after 30 sec, except H×2, which is the response to 0.04 μg/ml. Serpentine (10^{-8} and 10^{-7}) was added 30 sec before each histamine response between the bracket. Note potentiation of the histamine response.

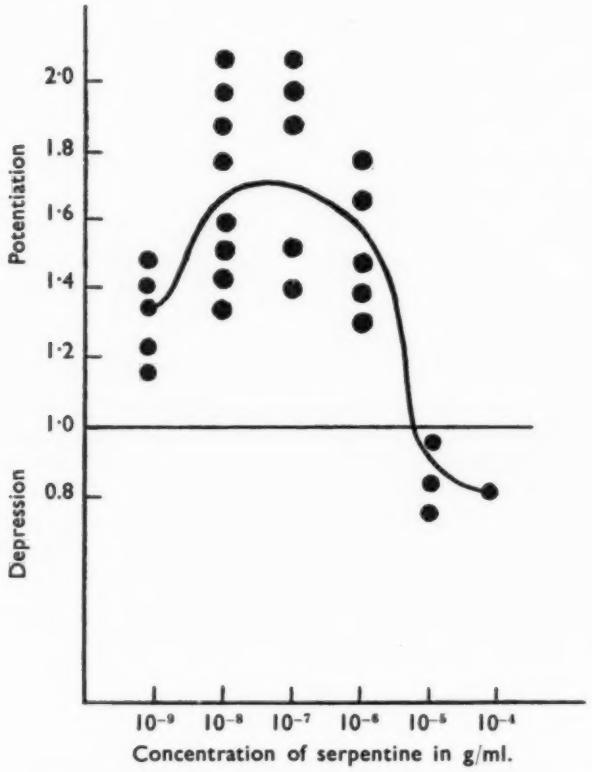

Fig. 2. Potentiation and depression of histamine response of guinea-pig ileum plotted against concentrations of serpentine. Measurement of each potentiation or depression in each experiment, represented by a dot, was made in terms of an equivalent dose of histamine.

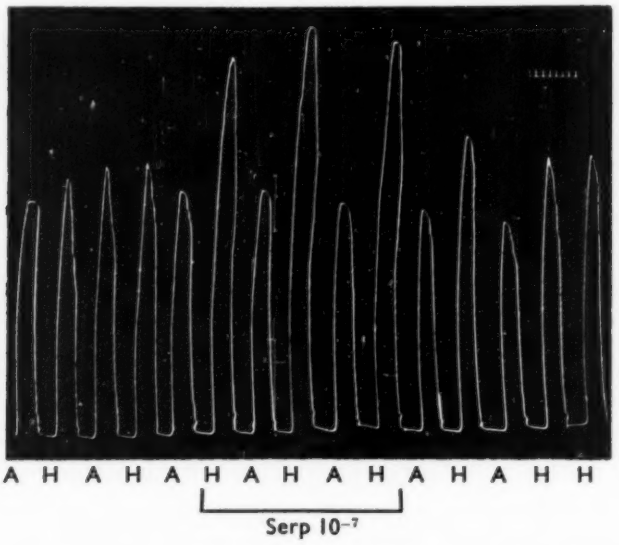

Fig. 3. Responses of guinea-pig ileum to acetylcholine (A) 0.2 μg/ml., histamine (H) 0.02 μg/ml. Serpentine 10^{-7} potentiated only histamine responses.

10^{-6} of serpentine raised the base line tone and depressed the histamine response. Fresh pieces of guinea-pig ileum showed better potentiation than ones kept in the refrigerator. The potentiation and depression of the histamine response on guinea-pig ileum by various concentrations are shown in Fig. 2.

Atropinization of ileum did not affect the potentiation. Alternating equipotent responses of acetylcholine and histamine were differentially affected by serpentine ; while histamine responses were potentiated, the acetylcholine responses were not affected or sometimes depressed (Fig. 3).

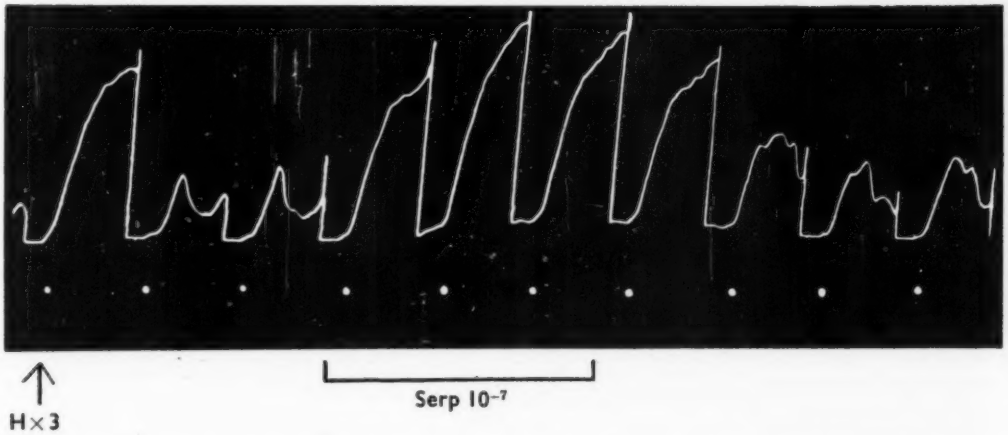

Fig. 4. Isolated guinea-pig uterus. At all white dots a response to 0.2 μg/ml. histamine, except H×3, where 0.6 μg/ml. histamine was given. 10^{-7} serpentine added 1 min before histamine response potentiated the response by more than three times.

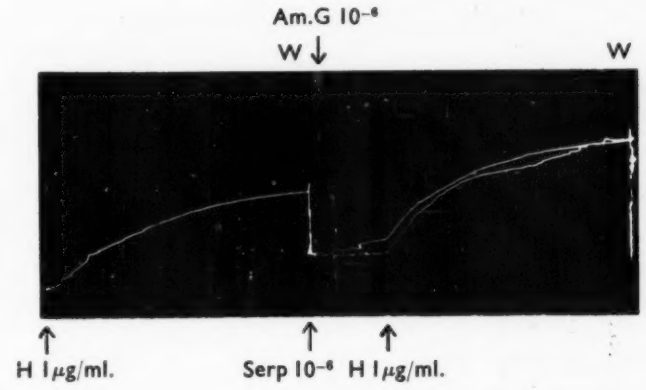

Fig. 5. Tracheal chain of guinea-pig. Three responses to 1 μg/ml. histamine (H). The first is a control. The latter two responses are superimposed. For 4 min before the lower response, the tissue was exposed to 10^{-6} g/ml. of serpentine (Serp). After eliciting the potentiated response of histamine and repeated washing until histamine responses returned to control level, the drum was moved back at 4 min before the upper superimposed response to histamine, aminoguanidine (Am.G) was added, to get a concentration of 10^{-6} in the bath. Note that serpentine and aminoguanidine produce equivalent potentiations.

Guinea-pig uterus. As indicated by Arunlakshana, Mongar & Schild (1954), only a few preparations could be stabilized. Minimal potentiation of histamine responses was obtained with 10^{-8} of serpentine. Maximal potentiation (three- or four-fold) occurred with concentrations of serpentine between 10^{-7} and 10^{-6} (Fig. 4). Higher concentrations produced marked rhythmic activity of the uterus.

Guinea-pig tracheal chain. Minimal potentiation of the histamine response was obtained with 10^{-8} of serpentine. 10^{-7} and 10^{-6} of serpentine produced the maximal potentiation (Fig. 5). Maximum potentiation obtained was 4 times the histamine response. Higher concentrations raised the base line and depressed the histamine response.

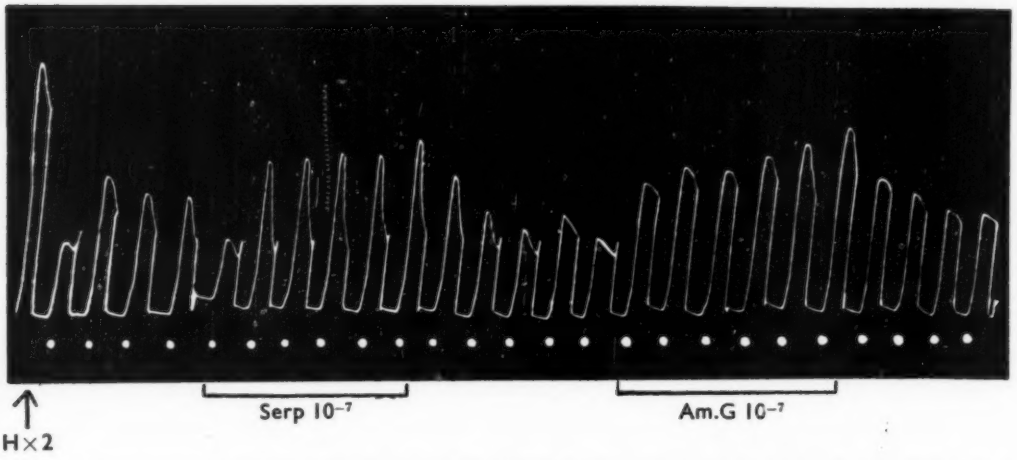

Fig. 6. Responses of guinea-pig ileum to 0.02 μg/ml. histamine given at white dots. H×2 is a response to 0.04 μg/ml. histamine. Serpentine (Serp 10^{-7}) and aminoguanidine (Am.G 10^{-7}) added at the bars. Note that there is a similar potentiation of histamine response after serpentine and aminoguanidine.

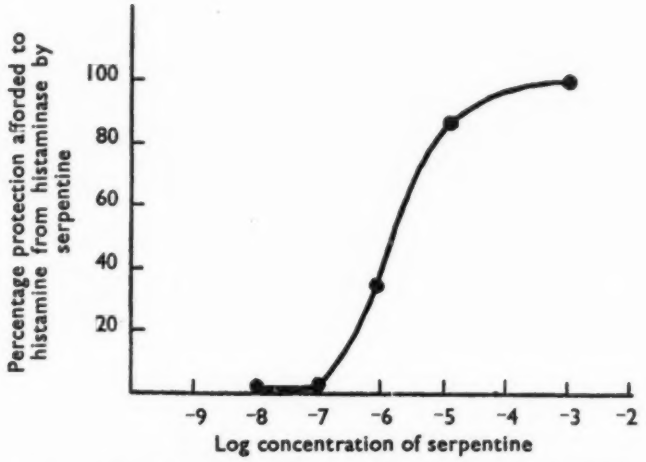

Fig. 7. The inhibition of histaminase by serpentine *in vitro*.

Comparison of serpentine and aminoguanidine. The potentiating activity of serpentine was compared with that of aminoguanidine on guinea-pig ileum and tracheal chain. Each comparative study was performed on the same strip of tissue. It was seen that the potentiation produced by equal concentrations of aminoguanidine and serpentine was approximately the same, on both guinea-pig ileum and tracheal chain. For comparisons only the concentrations producing the maximal potentiation were used (that is, 10^{-7} and 10^{-6}) (Figs. 5 and 6).

In vitro *experiments.* Aerobic incubation of histaminase with histamine (10^{-6}) at 37° C with and without various concentrations of serpentine showed that the minimal concentration of serpentine showing inhibition of histaminase activity was 10^{-6}. With higher concentrations of serpentine the inhibition of histaminase rose steeply. The protection afforded to histamine by serpentine when plotted against the concentration of serpentine showed an S-shaped curve (Fig. 7). Anaerobic incubation of histaminase with histamine produced no destruction of histamine.

DISCUSSION

We have demonstrated that serpentine potentiates histamine responses of guinea-pig ileum, uterus, and tracheal chain, organs shown by Arunlakshana, Mongar & Schild (1954) to be rich in histaminase. This potentiation is specific, as acetylcholine responses are not affected by serpentine. The potentiating activity of serpentine is of the same order of magnitude as that of aminoguanidine, which is a potent antihistaminase drug. Serpentine also inhibits the histamine-inactivating action of histaminase (Torantil), *in vitro.*

Antihistaminase activity has been described in various types of compounds. Many are carbonyl reagents (Arunlakshana, Mongar & Schild, 1954). The indole grouping has been implied to possess antihistaminase activity by Tabachnick & Roth (1957). Although it is present in both serpentine and reserpine, the latter does not show any antihistaminase activity. Reserpine neither potentiates the histamine response of guinea-pig ileum nor inhibits histaminase *in vitro* (unpublished observations). In fact it depresses the histamine responses of guinea-pig ileum.

Therapy with Rauwolfia serpentina is known to cause some histamine-like side effects such as nasal congestion, increased gastric acidity, and bronchoconstriction. An interesting hypothesis to explain the increased gastric acidity produced by antihistaminases in spite of the absence of histaminase in gastric mucosa has been put forward by Irvine, Duthie, Ritchie & Waton (1959). But since reserpine, which has no antihistaminase activity, produces increased gastric secretion, nasal stuffiness and bronchoconstriction, it is not reasonable to attribute the side effects of Rauwolfia therapy to the antihistaminase activity of serpentine. However, in serpentine we have a very potent antihistaminase drug which may be of use in experimental work.

We are thankful to Dr. B. P. Ghosh, of Gluconate Co., Calcutta, and Shri S. P. Bapat, of Hoechst Pharma., Bombay, for the liberal supplies of crystalline serpentine and Torantil respectively.

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REFERENCES

ARUNLAKSHANA, O., MONGAR, J. L. & SCHILD, H. O. (1954). Potentiation of pharmacological effects of histamine by histaminase inhibitors. *J. Physiol.* (*Lond.*), **123**, 32–54.

CASTILLO, J. C. & DE BEER, E. J. (1947). The tracheal chain. I. A preparation for the study of antispasmodics with particular reference to bronchodilator drugs. *J. Pharmacol. exp. Ther.*, **90**, 104–109.

CHATTERJEE, M. L. & HAUSLER, H. F. (1955). Anti-acetylcholine and anti-histamine actions of the total alkaloids of *Rauwolfia serpentina* Benth. *Nature* (*Lond.*), **176**, 701.

IRVINE, W. T., DUTHIE, H. L., RITCHIE, H. D. & WATON, N. G. (1959). The liver's role in histamine absorption from the alimentary tract. Its possible importance in cirrhosis. *Lancet*, **1**, 1064–1068.

LINDELL, S. E. & WESTLING, H. (1954). Potentiation of histamine effects by an antihistaminase. *Acta physiol. scand.*, **32**, 230–237.

SACHDEV, K. S. (1958). Some aspects of the pharmacology of serpentine with special reference to its antihistaminase activity. Dissertation accepted by Poona University, India, as part of M.D. pharmacology examination.

SACHDEV, K. S. & AIMAN, RANITA (1957). Potentiation of histamine response of guinea-pig ileum by serpentine. *Curr. Sci.*, **26**, 356–357.

TABACHNICK, I. I. A. & ROTH, F. E. (1957). The potentiation of histamine by imidazoleacrylcholine (murexine) and imidazolepropionylcholine (dihydromurexine). *J. Pharmacol. exp. Ther.*, **121**, 191–198.

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EFFECTS ON AMINE OXIDASE OF SUBSTANCES WHICH ANTAGONIZE 5-HYDROXYTRYPTAMINE MORE THAN TRYPTAMINE ON THE RAT FUNDUS STRIP

BY

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Certain substances, 2-bromolysergic acid diethylamide, dimethyltryptamine (3-(2-dimethylaminoethyl)indole), 2-methyldimethyltryptamine (3-(2-dimethylaminoethyl)-2-methylindole), and 5-benzyloxydimethyltryptamine (5-benzyloxy-3-(2-dimethylaminoethyl)indole), antagonize the effects of 5-hydroxytryptamine on the rat fundus strip more than those of tryptamine. These substances have been tested for their ability to inhibit the oxidation of tryptamine and 5-hydroxytryptamine by suspensions of guinea-pig liver and rat fundus. 2-Bromolysergic acid diethylamide has virtually no inhibitory activity and it is doubtful if the others produce any significant inhibition of amine oxidase in the concentrations which antagonize the effects of 5-hydroxytryptamine more than those of tryptamine. It seems that the differential character of the blocking action of these compounds should be ascribed either to interference with the transport of tryptamine (but not 5-hydroxytryptamine) through the cell wall, coupled with the block of a receptor common to both tryptamine and 5-hydroxytryptamine, or to the existence of separate tryptamine and 5-hydroxytryptamine receptors.

The amine oxidases of the guinea-pig liver and rat fundus appear to be a mixture of at least two types of enzyme, one of which has a higher affinity for 5-hydroxytryptamine than the other and is more susceptible to inhibition by 2-methyldimethyltryptamine.

Woolley & Shaw (1957) found that certain compounds (notably 1-benzyl-3-(2-dimethylaminoethyl)-5-methoxy-2-methylindole, " BAS ") antagonized the effects of 5-hydroxytryptamine more than those of tryptamine on the blood-pressure of the anaesthetized dog. They suggested that this substance blocked " serotonin-receptors " more than " tryptamine-receptors."

Barlow & Khan (1959a and b) found that certain analogues of tryptamine (notably 3-(2-dimethylamininoethyl)indole " dimethyltryptamine," 3-(2-dimethylaminoethyl)-2-methylindole " 2-methyldimethyltryptamine " and 5-benzyloxy-3-(2-dimethylaminoethyl)indole " 5-benzyloxydimethyltryptamine ") and also 2-bromolysergic acid diethylamide antagonized the effects of 5-hydroxytryptamine more than those of tryptamine on the rat fundus strip preparation described by Vane (1957).

Vane (1959) suggested that in the rat fundus strip tryptamine was destroyed inside the cell by amine oxidase because its action was potentiated by iproniazid (which he assumed to be acting specifically as an inhibitor of amine oxidase). The action of 5-hydroxytryptamine, however, was not potentiated by iproniazid, and it was suggested that this substance, unlike tryptamine, did not penetrate the cell.

Vane demonstrated the destruction of tryptamine and 5-hydroxytryptamine by ground rat fundus and the relative insolubility of 5-hydroxytryptamine in olive oil.

The greater antagonism of 5-hydroxytryptamine than of tryptamine, observed by Barlow & Khan, might be explained by supposing the existence of separate tryptamine and 5-hydroxytryptamine receptors: another possibility might, however, be that the differential blocking substances act at a common receptor and simultaneously penetrate the cell and inhibit amine oxidase (so preserving tryptamine from destruction). Govier, Howes & Gibbons (1953) had tested some analogues of 5-hydroxytryptamine, rather similar to those studied by Barlow & Khan, as substrates of the oxidases of guinea-pig liver and as inhibitors of the oxidation of tyramine. Although their results suggested that these differential blocking compounds might be inhibitors of amine oxidase, there was insufficient information to be certain about this.

This paper describes an attempt to investigate the problem manometrically. It was recognized that it would not be sufficient merely to demonstrate the ability or inability of the compounds to inhibit amine oxidase; it would be necessary to establish whether or not the substances were likely to have any effects on the particular enzyme (or enzymes) present in the rat fundus at the concentrations used in the pharmacological experiments. In manometric experiments the amounts of both enzyme and substrate are necessarily much larger than those in pharmacological experiments. If the degree of saturation of the enzyme is the same in both situations, however, the effects of inhibitors will be comparable. The experimental work began, therefore, with a study of the effects of substrate concentration on the rate of uptake of oxygen. Unfortunately the rat fundus is a poor source of oxidizing enzymes; usually almost a whole fundus was required per manometer flask and the range of substrate concentrations which could be tested was small. The effects of the substrates (tyramine, tryptamine and 5-hydroxytryptamine), and of the inhibitors, have consequently been studied mostly with acetone-powdered guinea-pig liver suspensions. Selected experiments, regarded as the most important, were then repeated using suspensions of ground rat fundus.

Ground rat uterus in oestrus has also been tested as an enzyme source. On the isolated rat uterus preparation, in contrast to the rat fundus, the effects of 5-hydroxytryptamine and of tryptamine were blocked to an equal extent by all the compounds (Barlow & Khan, 1959b), and it was expected that this tissue would be deficient in amine oxidase activity.

METHODS

Compounds

Tryptamine hydrochloride and tyramine hydrochloride were obtained from British Drug Houses, 5-hydroxytryptamine creatinine sulphate monohydrate from May & Baker, iproniazid (Marsilid phosphate) from Roche Products, 2-bromolysergic acid diethylamide from Sandoz, and the remaining compounds were synthesized (Barlow & Khan, 1959a, b).

Tissues

Guinea-pig liver. An acetone-powder was made from the livers of a number of guinea-pigs and stored at room temperature and atmospheric pressure over calcium chloride. When required for use, a quantity was weighed out, suspended in phosphate buffer (0.067 M; *p*H

7.4) and centrifuged for a few min in a "Wifug" bench centrifuge at 3,500 rev/min. The supernatant was decanted off and the sediment re-suspended and centrifuged again. After further re-suspension and centrifugation the sediment was suspended once more and the volume made up to the desired amount (usually 100 mg/ml. or 50 mg/ml.).

Rat fundus. This is a tough structure which cannot be broken up easily. An "Atomix" blender only reduced it to a fibrous mat and it was extremely tedious to break down any appreciable quantity of material by hand in an all-glass homogenizer (the only other equipment available). The fundus was therefore dissected, washed in phosphate buffer (0.06 M, *p*H 7.4), dried with filter paper, weighed, cut as small as possible with scissors and ground with sand in phosphate buffer. The suspension was made up to a measured volume and used directly.

Rat uterus. On the day before the experiment, the rats received an injection of stilboestrol (10 μg/0.1 ml. arachis oil/100 g body weight) in order to induce oestrus. The rat uterus was dissected out and ground with sand in phosphate buffer exactly as with the rat fundus (it was much easier to break down than the fundus).

Variation in the activity of the enzyme source

In experiments with the guinea-pig liver suspensions, the acetone powder should provide a homogeneous source of enzyme for use in a number of experiments. Different batches of powder, however, varied in activity. In this work only 4 batches were used; the last, in particular, was less active than the others. The suspensions of liver in phosphate buffer, even when stored at 4°, deteriorated detectably in 24 hr, and usually a fresh suspension was made every day.

In experiments with ground rat fundus, sufficient material (usually from 5 to 10 funduses) was ground together to provide a source for a particular experiment, but fresh material (possibly, therefore, of different activity) had to be employed each day. This was also necessary in experiments with rat uterus.

Procedure

In all the manometric experiments standard procedures were employed. The flasks contained 0.3 ml. N potassium hydroxide in the centre well, pure oxygen was used and, unless otherwise stated, the temperature was 37° C. Thermobarometers, and suitable blank experiments incorporating cell fragment suspensions but no substrate, or cell fragments and inhibitor but no substrate, were included whenever necessary. The quantity of tissue per flask and the time during which the inhibitor and enzyme were left in contact was recorded.

In experiments with guinea-pig liver the amount of tissue per flask was usually 50 mg, but in some experiments it was greater (up to 100 mg). In experiments with rat fundus the amount varied from 180 mg to 320 mg/flask (being the same for each flask in any one set of experiments and usually around 220 mg); with the uterus it varied from 170 to 260 mg (usually around 220 mg). The actual quantity used depended partly on the efficiency of the material as a catalyst of oxidation; the aim was to obtain constant results with the standard concentrations of substrate.

In experiments with substrates at a concentration of 10^{-3} M the total volume in the flask was 4.0 ml.; at a concentration of 2×10^{-3} M it was 3.0 ml.; and at higher concentrations, 2.0 ml.

Evaluation of results

Rate of oxidation. In experiments with guinea-pig liver, readings were taken 3, 6, 9, 12, 15, 20, 25 and 30 min after tipping. A graph of the net oxygen uptake (deducting blank values for uptake in the absence of substrate) was plotted for each flask containing substrate. In the early part of the experiment, this graph should be linear and indicate only the reaction of oxygen with amine rather than any further oxidation of products such as aldehydes. From the slope of this line the uptake (in μl.) over a period of 9 min was calculated.

In experiments with ground rat fundus and rat uterus the reaction was much slower. Readings were taken 5, 10, 15, 30, 45 and 60 min after tipping and the oxygen uptake was expressed as μl./g tissue (wet weight)/hr.

Effects of inhibitors. The effect of (a particular concentration of) an inhibitor was estimated as the percentage inhibition of the oxidation of (a particular concentration of) substrate. The net oxygen uptake of the flask containing inhibitor was compared with the net oxygen uptake of the flask containing the same concentration of substrate without the inhibitor. The average was usually taken of the values obtained from the figures 15 and 30 min after tipping. When, however, these values differed considerably (as they did when only small volumes of oxygen were involved as in the experiments with rat fundus) it was necessary to plot the two graphs of oxygen uptake against time and to estimate the percentage inhibition by comparing their slopes.

RESULTS

Variation of rate of uptake of oxygen with substrate concentration

Guinea-pig liver. Table 1 and Fig. 1 summarize the results of the first group of experiments. The curve for tyramine indicates that it is the best substrate. The curves for tryptamine and 5-hydroxytryptamine are indistinguishable and flatter than the curve for tyramine. It was concluded that a concentration of 10^{-3} M of any of these substrates would produce something approaching half-saturation of the enzyme. This conclusion was reasonably well confirmed on repetition: Table 2 shows results of similar experiments with a less active acetone-powder performed

TABLE 1

OXIDATION OF SUBSTRATES BY SUSPENSIONS OF GUINEA-PIG LIVER CELL FRAGMENTS

Figures show the oxygen uptake in μl. from the 3rd to the 12th min after tipping. The number of experiments is shown in brackets and the mean value is given with the standard error. The oxygen uptake has been corrected for thermobarometer changes and uptake by tissue in the absence of substrate. In all experiments each flask contained 50 mg tissue. The acetone powder used was dated September 16, 1959

	Molar concentration				
Substrate	10^{-3}	2×10^{-3}	5×10^{-3}	10^{-2}	2×10^{-2}
Tyramine	18·2±1·1 (5)	22·6±0·9 (5)	28·8±2·3 (5)	31·6±2·8 (5)	28·8±2·2 (6)
Tryptamine	12·8±0·7 (6)	13·6±1·4 (5)	19·2±1·5 (5)	21·6±1·2 (5)	22·8±1·0 (5)
5-Hydroxytryptamine	12·1±0·7 (8)	14·1±0·6 (8)	19·0±1·3 (6)	19·2±1·2 (6)	

TABLE 2

OXIDATION OF SUBSTRATES BY SUSPENSIONS OF GUINEA-PIG LIVER CELL FRAGMENTS

As Table 1, but in all experiments each flask contained 70 mg tissue and the acetone powder was dated September 7, 1960

	Molar concentration				
Substrate	10^{-3}	2×10^{-3}	5×10^{-3}	10^{-2}	2×10^{-2}
Tyramine	18·4±1·4 (4)	21·0±3·7 (3)	23·1±0·4 (3)	25·9±2·3 (5)	25·9±1·9 (4)
Tryptamine	9·4±0·6 (6)	12·0±2·0 (2)	13·2±0·6 (6)	14·0±1·5 (2)	13·2±0·2 (2)
5-Hydroxytryptamine	9·3±0·7 (6)	9·5±0·5 (2)	13·1±0·6 (6)	13·3±0·3 (3)	7·7±1·2 (2)

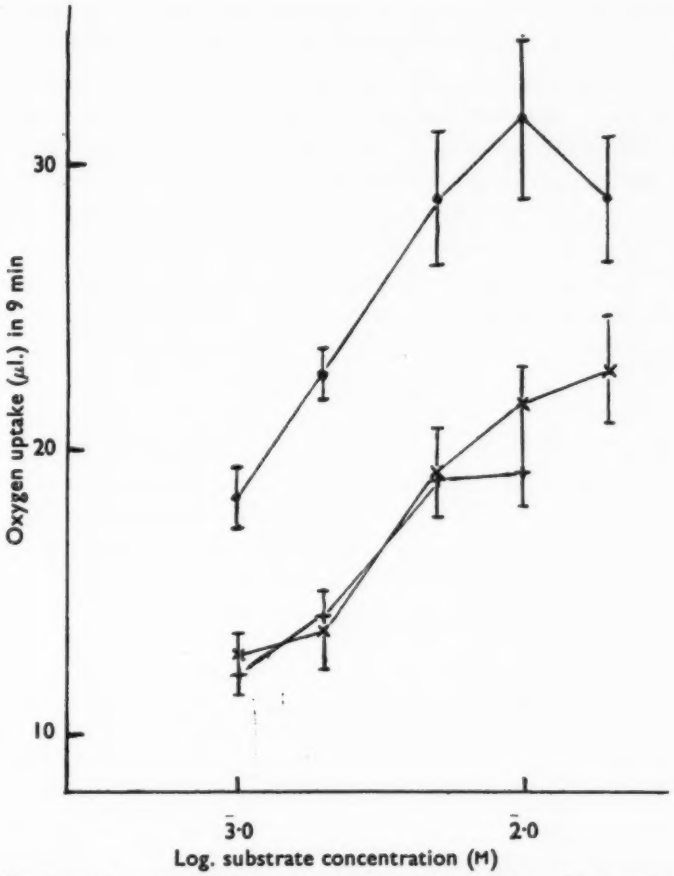

Fig. 1. Effect of substrate concentration on rate of oxygen uptake with suspensions of acetone-powdered guinea-pig liver. Each flask contained 50 mg tissue. Ordinate: oxygen uptake μl. in 9 min. Abscissa: log. molar concentration of substrate. ● Tyramine. X Tryptamine. + 5-Hydroxytryptamine.

12 months later. 5-Hydroxytryptamine was tested as the creatinine sulphate, but creatinine itself, even in a concentration of 10^{-2} M, did not take up oxygen in the presence of guinea-pig liver suspension, nor did it inhibit at all the oxidation of tyramine.

TABLE 3

OXIDATION OF SUBSTRATES BY SUSPENSIONS OF GROUND RAT FUNDUS

Figures show the oxygen uptake in μl./g tissue (wet weight)/hr. The number of experiments is shown in brackets and the mean value is given with the standard error. The oxygen uptake has been corrected for thermobarometer changes and uptake by tissue in the absence of substrate. Results of Vane (1959) using a substrate concentration of $7{\cdot}5\times10^{-3}$ M were: tryptamine, 124; 5-hydroxytryptamine, 142; tyramine, 207

	Molar concentration	
Substrate	5×10^{-3}	10^{-2}
Tryptamine	73·5±7·7 (6)	124±15·4 (9)
5-Hydroxytryptamine	224±12·9 (6)	196±14·3 (4)
Tyramine		271±16·9 (9)

Ground rat fundus. The results of experiments with the ground rat fundus are summarized in Table 3. Only 2 substrate concentrations were used. When the concentration was less than 5×10^{-3} M the oxygen uptake was too small to be estimated accurately: a concentration of 5-hydroxytryptamine greater than 10^{-2} M was difficult to obtain because a 2×10^{-1} M solution of this compound is almost saturated and this had to be diluted by the volume of the suspension of tissue.

The results suggest that a concentration of 10^{-2} M 5-hydroxytryptamine saturates the amount of enzyme in these experiments but that tryptamine at this concentration does not. The decrease in oxygen uptake with the higher concentration of 5-hydroxytryptamine resembles the results with the guinea-pig liver, particularly those shown in Table 2, where a 5×10^{-2} M solution was tested.

Inhibitory activity

Guinea-pig liver. Preliminary experiments, summarized in Table 4, produced two surprising results. First, 2-bromolysergic acid diethylamide appeared to be almost inactive, and, second, the tryptamine analogues appeared to inhibit the oxidation of tyramine more than that of tryptamine. It was because of these results that it was realized that the effect of concentration on the rate of oxygen uptake would have to be studied for each particular substrate.

TABLE 4

EFFECTS OF INHIBITORS ON OXIDATIONS BY SUSPENSIONS OF GUINEA-PIG LIVER AT 37° C

Figures in brackets indicate the number of experiments: the mean is given with the standard error

		% Inhibition of oxidation of	
Compound	Molar concn.	Tyramine (10^{-2} M)	Tryptamine (10^{-2} M)
2-Bromolysergic acid diethylamide	5×10^{-4}	28	
	10^{-4}	0	
Iproniazid	10^{-5}	23	
Dimethyltryptamine	10^{-3}	79·7±8·1 (3)	44·0±3·5 (3)
2-Methyldimethyltryptamine	10^{-3}	58·3±12·0 (3)	28·7±14·1 (3)
5-Benzyloxydimethyltryptamine	10^{-3}	50·7±2·7 (3)	16·3±3·3 (3)

The results of the main group of experiments are summarized in Table 5. While the accuracy of single experiments is uncertain, it should be noted that with two exceptions (marked) the antagonists were less effective when diluted and that they were more effective in inhibiting the oxidation of 10^{-3} M substrate than of 5×10^{-3} M. From these results it was concluded that the tryptamine analogues, particularly 2-methyldimethyltryptamine, but not iproniazid, really did inhibit the oxidation of 5-hydroxytryptamine more than that of tryptamine. These results were confirmed when checked subsequently (Table 5) and 2-bromolysergic acid diethylamide was found to be completely without effect on the oxidation of tryptamine, 5-hydroxytryptamine, or tyramine in the highest concentrations which could be tested (5×10^{-3} M), even when left in contact with the suspension more than 30 min before adding the substrate.

TABLE 5

EFFECTS OF INHIBITORS ON OXIDATIONS BY SUSPENSIONS OF GUINEA-PIG LIVER AT 37° C

Figures in brackets indicate the number of experiments: the mean is given with the standard error. An asterisk indicates that the result does not conform with the expectation that inhibition should increase as the inhibitor concentration is increased or the substrate concentration decreased. The second set of results for 2-methyldimethyltryptamine and 2-bromolysergic acid diethylamide were obtained approximately twelve months after the results in the rest of this table

		% Inhibition of oxidation of			
		Tryptamine		5-Hydroxytryptamine	
	Molar concn.	10^{-3} M	5×10^{-3} M	10^{-3} M	5×10^{-3} M
Dimethyltryptamine	10^{-3}	65	46	100	94
	2×10^{-4}	52·7±16·2 (3)	0	89·7±10·3 (3)	50
2-Methyldimethyl-tryptamine	10^{-3}	20*	27	99	100
	2×10^{-4}	20·3±10·7 (3)	0	88·0±7·1 (3)	42
5-Benzyloxydimethyl-tryptamine	10^{-3}	28*	26	60	46
	2×10^{-4}	36·5±2·5 (2)	—	49·5±9·5 (2)	—
Iproniazid	3×10^{-5}	71·0±12·4 (3)	58·7±12·9 (3)	52·0±23·7 (3)	36·3±5·7 (3)
2-Methyldimethyl-tryptamine	10^{-3}	27±7 (2)*	28±10 (2)	95±4 (2)	91±8 (2)
	2×10^{-4}	6±6 (2)	10±10 (2)	86±1 (2)	64±1 (2)
2-Bromolysergic acid diethylamide	5×10^{-4}	0	0	0	0

Ground rat fundus. Results of experiments with 2-methyldimethyltryptamine, iproniazid and 2-bromolysergic acid diethylamide on suspensions of ground rat fundus are shown in Table 6. As in the experiments with guinea-pig liver, 2-methyldimethyltryptamine inhibited the oxidation of 5-hydroxytryptamine more

TABLE 6

EFFECTS OF INHIBITORS ON OXIDATIONS BY SUSPENSIONS OF GROUND RAT FUNDUS AT 37° C

Figures in brackets indicate the number of experiments : the mean is given with the standard error

		% Inhibition of oxidation of			
		Tryptamine		5-Hydroxytryptamine	
	Molar concn.	5×10^{-3} M	10^{-2} M	5×10^{-3} M	10^{-2} M
2-Methyldimethyl-tryptamine	2×10^{-4}	(9±9 (3)	3±3 (2)	91±5 (3)	75±7 (2)
Iproniazid	3×10^{-5}	56		63±15 (2)	
	10^{-5}	15±1 (2)		38±3 (2)	
2-Bromolysergic acid diethylamide	5×10^{-4}	0		22	

than that of tryptamine. In the highest concentration which could be tested, 5×10^{-4} M, 2-bromolysergic acid had little effect on the oxidation of tryptamine or 5-hydroxytryptamine even when left in contact with the suspension more than 30 min before adding the substrate.

Experiments with ground rat uterus

The results of these experiments are summarized in Table 7. Because the considerable ability of this tissue to catalyse the oxidation of tyramine and tryptamine was unexpected, the experiments were repeated at 30° C, the temperature at which the tissue is used in pharmacological experiments. Even at this temperature the oxygen uptake was considerably greater than with ground rat fundus (weight for weight). In two experiments at 37° C the oxidation of both tyramine and tryptamine (10^{-2} M) was completely inhibited by 10^{-5} M iproniazid.

TABLE 7

OXIDATION OF SUBSTRATES BY SUSPENSIONS OF RAT UTERUS IN OESTRUS

Figures show the oxygen uptake in μl./g tissue (wet weight)/hr. The number of experiments is shown in brackets and the mean value is given with the standard error. Compare these results with those in Table 3

Substrate		37° C	30° C
Tryptamine	10^{-2} M	378·9±22·9 (8)	240·8±41·4 (6)
Tyramine	10^{-2} M	602·2±27·2 (9)	427·3±6·9 (6)

DISCUSSION

In Table 8 are set out the approximate concentrations of the compounds which are effective in the pharmacological experiments and in inhibiting the oxidation of tryptamine by ground tissues. The figures for pharmacological experiments with iproniazid are based on the observations of Vane (1959), Barlow and Khan (1959b) and Khan (1959). Vane found that 3×10^{-5} M iproniazid potentiated the effects of tryptamine 12.7 times on the rat fundus strip; Khan recorded a 36-fold potentiation at this concentration. Barlow and Khan reported that 3×10^{-5} M iproniazid did not potentiate the action of tryptamine or 5-hydroxytryptamine on the rat uterus, but Khan recorded slight effects (not greater than a factor of 2) on the responses

TABLE 8

EFFECTIVE MOLAR CONCENTRATIONS

Compound	Modification of effects of tryptamine on: Rat fundus	Rat uterus	Inhibition of destruction of tryptamine by oxidases of: Guinea-pig liver suspensions	Rat fundus suspensions
Dimethyltryptamine	3×10^{-7}	100×10^{-7}	$1{,}000 \times 10^{-7}$	
2-Methyldimethyltryptamine	20	100	1,000	$1{,}000 \times 10^{-7}$
5-Benzyloxydimethyltryptamine	6	90	1,000	
Iproniazid	300	30–300	100	100
2-Bromolysergic acid diethylamide	1	0·1	>5,000	>5,000

to tryptamine with concentrations of iproniazid varying from 3×10^{-6} M to 3×10^{-5} M (allowed to act for 45 min). Because the figures in Table 8 showing the concentrations inhibiting the oxidation of tryptamine by ground tissues were obtained from experiments in which the enzyme or enzymes should not have been saturated it should be justifiable to assume that they would be comparable with the concentrations effective inside the cell in the pharmacological experiments. This

assumption receives support from the figures for iproniazid: the concentrations effective in inhibiting the oxidation of tryptamine by ground rat fundus are comparable with those which potentiate the action of tryptamine on the rat fundus strip.

2-Bromolysergic acid diethylamide, however, cannot have any effect on amine oxidase in the experiments with the rat fundus strip unless it is being concentrated inside the cell to a fantastic extent (to such an extent that it must almost form a saturated solution). For this reason the differential blocking action of 2-bromolysergic acid diethylamide cannot be ascribed to block of a common receptor coupled with a simultaneous inhibition of amine oxidase, although it might be supposed that potentiation of tryptamine is brought about by blocking its entry through the cell wall.

The results for the tryptamine analogues are more ambiguous. It is possible to suppose that these may be concentrated inside the cell and produce a differential block by, simultaneously, blocking a process activated by both tryptamine and 5-hydroxytryptamine, and inhibiting the destruction of tryptamine by amine oxidase. There are, however, objections to this idea: (1) The compounds would have to be concentrated about 100-fold inside the cell (that is, to a very much greater extent than iproniazid; there seems no reason why this should be so). (2) In the rat uterus conditions are more favourable to inhibition of amine oxidase than in the fundus (see Table 8), but the compounds antagonize tryptamine and 5-hydroxytryptamine to the same extent.

These results draw attention to the need for information about the effects of these compounds on the transport of tryptamine across the cell membrane. If 2-bromolysergic acid diethylamide, for instance, does not affect this, it seems unlikely that tryptamine and 5-hydroxytryptamine can be acting on a common receptor. It would also be gratifying to have direct evidence that 5-hydroxytryptamine does not penetrate the cells of the rat fundus, for if it does penetrate to any extent the tryptamine analogues used in this work should inhibit its destruction more than that of tryptamine. It appears, from the results, that the intracellular destruction of tryptamine by amine oxidase may be peculiar to the rat fundus; it does not seem likely to occur in the rat uterus.

The greater inhibition of the oxidation of 5-hydroxytryptamine than of tryptamine by suspensions of guinea-pig liver and ground rat fundus is an unexpected finding. It does not appear to be an artefact caused by working at different levels of saturation of the enzyme, for in the experiments with suspensions of guinea-pig liver the degree of saturation should be the same for both substrates. Hope & Smith (1960) have studied the substrate specificity of amine oxidases obtained from a variety of tissues of the mouse and deduced the existence of more than one species of enzyme. From the results of our experiments it would seem that the enzymes in guinea-pig liver and rat fundus are not exactly the same (compare the results for tryptamine and 5-hydroxytryptamine in Tables 1, 2 and 3). If there are different types of amine oxidase, it is conceivable that the enzymes in the liver and fundus are not homogeneous. It appears possible to distinguish 2 types; one, for which 5-hydroxytryptamine has a higher affinity than tryptamine, is more readily inhibited by 2-methyldimethyltryptamine than the other. The existence of

2 such types might account for the variation in the activity of different acetone-powders of guinea-pig liver or of suspensions of rat fundus and also for the impression that the ability of these to oxidize tryptamine seems to deteriorate more rapidly than ability to oxidize 5-hydroxytryptamine or tyramine.

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REFERENCES

BARLOW, R. B. & KHAN, I. (1959a). Actions of some analogues of tryptamine on the isolated rat uterus and on the isolated rat fundus strip preparations. *Brit. J. Pharmacol.*, **14**, 99–107.

BARLOW, R. B. & KHAN, I. (1959b). Actions of some analogues of 5-hydroxytryptamine on the isolated rat uterus and the rat fundus strip preparations. *Brit. J. Pharmacol.*, **14**, 265–272.

GOVIER, W. M., HOWES, B. G. & GIBBONS, A. J. (1953). The oxidative deamination of serotonin and other 3-(beta-aminoethyl)-indoles by monoamine oxidase and the effect of these compounds on the deamination of tyramine. *Science*, **118**, 596–597.

HOPE, D. B. & SMITH, A. D. (1960). Distribution and activity of monoamine oxidase in mouse tissues. *Biochem. J.*, **74**, 101–107.

KHAN, I. (1959). Drugs modifying the actions of 5-hydroxytryptamine. Ph.D. thesis, University of Edinburgh, p. 115.

VANE, J. R. (1957). A sensitive method for the assay of 5-hydroxytryptamine. *Brit. J. Pharmacol.*, **12**, 344–349.

VANE, J. R. (1959). The relative activities of some tryptamine analogues on the isolated rat stomach strip preparation. *Brit. J. Pharmacol.*, **14**, 87–98.

WOOLLEY, D. W. & SHAW, E. (1957). Differentiation between receptors for serotonin and tryptamine by means of the exquisite specificity of antimetabolites. *J. Pharmacol. exp. Ther.*, **121**, 13–17.

Brit. J. Pharmacol. (1961), **16**, 163–169.

SOME INHIBITORS OF HISTAMINE-INDUCED AND FORMALDEHYDE-INDUCED INFLAMMATION IN MICE

BY

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The swelling and capillary hyperpermeability of the mouse foot in response to an injection of formaldehyde, and the increased capillary permeability to an intradermal injection of histamine, have been investigated. Cortisone, mepyramine and sodium salicylate were effective in reducing histamine-induced inflammation, sodium salicylate being less active in the adrenalectomized animal. In formaldehyde-induced inflammation, however, cortisone was ineffective whereas sodium salicylate was effective in the intact mouse, but not in the adrenalectomized animal. Certain aryloxypropionates and anti-esterases were also active in reducing the severity of the formaldehyde-induced inflammation.

Formaldehyde will induce an inflammatory reaction at the site of injection, and this has been used by Parratt & West (1957) for studying inflammation quantitatively in the rat foot. Selye (1949) first described the effect of injected formaldehyde in the rat foot as an "arthritic" reaction, but this has been criticized by Bourne (1951) as inaccurate. Various authors are agreed, however, upon the main point, that formaldehyde produces a local inflammation.

The reaction of the rat foot to formaldehyde has been investigated pharmacologically by Parratt & West (1958), who showed that the reaction was not antagonized by inhibitors of either histamine or 5-hydroxytryptamine, with the exception of the compound methotrimeprazine, which is probably active because of some action separate from its antihistaminic action. Selye (1949) has shown that in the adrenalectomized rat both cortisone and adrenocorticotrophic hormone are effective in antagonizing the reaction to formaldehyde. It has been reported by Setnikar, Salvaterra & Temelcou (1959) that the swelling of the rat foot caused by an injection of formaldehyde can be partially prevented by iproniazid, phenylbutazone and salicylamide. Recently, Buch (1959) has shown that various phenylpropylcarbamates are effective in reducing the reaction to formaldehyde.

Histamine, having been implicated in the mediation of inflammation for many years, has been extensively studied. Lockett & Jarman (1958) have devised a satisfactory method for relating the response quantitatively to an intradermal injection of histamine in the mouse.

The present work shows that formaldehyde-induced inflammation in the mouse foot can be inhibited by a number of substances, some of which may also inhibit the reaction to an intradermal injection of histamine.

METHODS

Formaldehyde-induced inflammation in the mouse foot. Preliminary experiments showed that 0.05 ml. of a 3.5% w/v solution of formaldehyde in 0.9% sodium chloride produced a submaximal degree of swelling which was very considerably greater than that produced by the same vol. of 0.9% sodium chloride alone. Since there was slight tissue damage from the injection of 0.05 ml. of 0.9% sodium chloride one hind foot was injected with this and the opposite foot injected with the formaldehyde solution, so that the relative degrees of swelling could be compared in each animal. The injections were made subcutaneously into the dorsum of the foot, as this was found to produce less mechanical trauma than an injection beneath the plantar aponeurosis.

Adult mice weighing between 25 and 35 g, of both sexes, were arranged in groups of 5 animals. Those animals receiving pretreatment were given daily subcutaneous injections of the drug into the forearm in a dose vol. of 0.1 ml., except those receiving anti-esterases, which were given intramuscularly daily into the leg on the same side as was to be used for the injection of formaldehyde. The pretreatment was continued for 3 days, and the final injection was given 30 min before the injection of formaldehyde. Immediately before the injection of formaldehyde each mouse was given into the tail vein 0.1 ml. of a 1% w/v solution of Evans blue in 0.9% sodium chloride. The extent of the swelling and the leakage of blue dye were noted by two observers at the end of 1 hr from the time of giving the formaldehyde, and given a subjectively assigned score of from 0 to 3. The mean response of the members of each group was then calculated for both the swelling and the blue dye leakage, and expressed in each case as a percentage of that seen in a control group which was given pretreatment with 0.9% sodium chloride for 3 days.

Since it was doubtful whether these subjectively assigned values for foot swelling could be related to objectively made measurements such as foot volume or ankle joint diameter, we made some observations using a fluid displacement method for determining the foot vol. and found that the results were comparable with the subjectively assigned results.

Histamine-induced inflammation in the abdominal skin of mice. Mice were arranged in groups as above and an intradermal injection of 2 μg of histamine in 0.1 ml. of 0.9% sodium chloride was made into ventral abdominal skin. The procedure was that of Lockett & Jarman (1958), with the difference that we did not anaesthetize the animal to give the histamine. Not only is this more convenient, but it removes the objection that the anaesthesia may alter capillary permeability, as in fact was demonstrated by Lockett & Jarman (1958). After 30 min the mice were killed, the ventral abdominal skin reflected, and the intensity of the blue dye leakage on the inside of the skin noted. For each animal the coloration was given a subjectively assigned score of from 0 to 3, so that the mean response for each group could be calculated and the result expressed as a percentage of the response in the control group of animals pretreated with normal saline.

Adrenalectomized mice. Mice were subjected to a bilateral adrenalectomy under ether anaesthesia, using the dorsal approach. The animals were maintained post-operatively by administration of 2 mg/kg/day of cortisone acetate given intramuscularly. The mice were also given 0.9% sodium chloride in place of drinking water.

Chemicals. Formaldehyde was reagent grade material protected from light and contained no free acid. Other drugs used were histamine diphosphate, mepyramine maleate, a microcrystalline suspension of cortisone acetate, atropine sulphate, adrenaline bitartrate, physostigmine salicylate, edrophonium chloride, neostigmine bromide, di*iso*propylfluorophosphonate in arachis oil, sodium salicylate, phenylbutazone, sodium 2:3-dihydroxybenzoate (sodium gentisate), a suspension of 3-(*o*-methoxyphenoxy)-2-hydroxypropyl carbamate (methocarbamol), sodium alpha-4-*sec*-butylphenoxypropionate (B.C. 7586), sodium alpha-4-phenylphenoxypropionate (B.C. 8402), sodium alpha-4-carboxyphenoxypropionate (B.C. 8424s), sodium alpha-4-hydroxyphenoxypropionate (B.C. 8422s), tolazoline hydrochloride, ephedrine hydrochloride, freshly prepared sodium acetylsalicylate, a suspension of amidopyrine and a suspension of N-acetyl-p-aminophenol.

RESULTS

Formaldehyde-induced inflammation in the mouse foot. The results of the experiments which were performed to determine the effectiveness of antagonists to formaldehyde-induced inflammation are presented in Tables 1 and 2. Sodium

TABLE 1

ANTAGONISTS OF FORMALDEHYDE-INDUCED INFLAMMATION

(Normal intact mice)

An asterisk denotes that the difference between the response of a group and that of the control group is statistically significant ($P<0.05$)

Drug	Daily dose mg/kg	Mean inflammatory response at 1 hr as a % of the control: Blue colour	Swelling
Normal saline	—	100	100
Phenylbutazone	75	28*	33*
Cortisone acetate	40	86	99
Sodium gentisate	75	87	91
Methocarbamol	75	23*	25*
B.C. 8402	75	67*	54*
B.C. 8422s	75	46*	58*
B.C. 8424s	75	68*	50*
Sodium acetylsalicylate	150	70*	66*
N-Acetyl-p-aminophenol	150	89	77*
Amidopyrine	75	71*	57*
Atropine sulphate	40	104	95
Atropine sulphate + Physostigmine	40 + 0·4	86	74*
Atropine sulphate + Neostigmine	40 + 0·1	111	96
Atropine sulphate + Edrophonium	40 + 4	113	107
Tolazoline	0·1	73*	72*
Tolazoline + B.C. 7586	0·1 + 75	68*	56*
Adrenaline	0·4 (single dose)	109	91
Ephedrine	2	83	75*

TABLE 2

ANTAGONISTS OF FORMALDEHYDE-INDUCED INFLAMMATION

(Adrenalectomized mice=A. Normal intact mice=N)

An asterisk denotes that the difference between the response of a group and that of the control group is statistically significant ($P<0.05$)

Drug	Daily dose mg/kg		Mean inflammatory response at 1 hr as a % of the control: Blue colour	Swelling
Sodium salicylate	150	N.	75*	73*
Sodium salicylate	150	A.	96	102
B.C. 7586	75	N.	53*	61*
B.C. 7586	75	A.	79*	82*
Atropine sulphate + Di*iso*propylfluorophosphonate	40 + 2	N.	32*	48*
Atropine sulphate + Di*iso*propylfluorophosphonate	40 + 2	A.	41*	67*
Normal saline	—	A.	127*	115*

salicylate and N-acetylaminophenol were slightly active, whereas cortisone acetate was inactive. Methocarbamol, amidopyrine and phenylbutazone, however, were highly active, whereas the four aryloxypropionates tested and sodium acetylsalicylate were moderately active. The activity of phenylbutazone confirmed the observations of Setnikar, Salvaterra & Temelcou (1959). In the cortisone-maintained adrenalectomized mice both sodium salicylate and B.C. 7586 were less effective than in the intact animals.

Several compounds with anti-esterase properties were tested for anti-inflammatory activity in fully atropinized mice, since they were administered in a dose which would otherwise cause considerable disturbance from the inhibition of cholinesterase. Atropine in the dose used had no anti-inflammatory action of its own. Di*iso*propylfluorophosphonate had a very marked inhibitory effect against the reaction caused by formaldehyde, as also had physostigmine, but to a less extent. Both neostigmine and edrophonium were inactive in this test in the doses used, which were also approximately the maximum tolerated doses. Spector & Willoughby (1960) have reported that di*iso*propylfluorophosphonate will antagonize the production of a turpentine pleurisy in rats, but in their test physostigmine was inactive. Since both the species and the nature of the inflammation were different in their work it is not possible to make a valid comparison between their results and ours.

Adrenaline has been reported by Geschickter, O'Malley & Rubacky (1960) to inhibit the inflammatory reaction to the injection of egg-white, and as there is some evidence (Smith, 1955) that salicylates can cause a stimulation of the adrenal medulla, it seemed worth while attempting to investigate the possibility that the aryloxypropionates and the other analgesic-antipyretics were acting through the adrenal medulla.

TABLE 3

ANTAGONISTS OF HISTAMINE-INDUCED CAPILLARY HYPERPERMEABILITY
(Normal = N; adrenalectomized = A)

An asterisk denotes that the difference between the response of a group and that of the control group is statistically significant ($P < 0.05$)

Drug	Daily dose mg/kg		Mean inflammatory response at 30 min as a % of the control
Normal saline	Control	N.	100
Normal saline	—	A.	96
Sodium salicylate	150	N.	36*
Sodium salicylate	150	A.	79*
B.C. 7586	75	N.	98
B.C. 8402	75	N.	105
Cortisone acetate	40	N.	47*
Mepyramine maleate	5 (single dose)	N.	23*

Tolazoline was chosen as an antagonist to the peripheral actions of adrenaline, but instead of blocking the action of B.C. 7586 it can be seen (Table 1) that tolazoline had some inhibitory action to formaldehyde-induced inflammation. This point is being investigated further. Adrenaline was not an effective antagonist, but since we have shown some activity by ephedrine it seems probable that the failure of adrenaline is due to its being more rapidly inactivated in the body than ephedrine.

Histamine-induced inflammation in the mouse skin. The results shown in Table 3 indicate that sodium salicylate is capable of inhibiting the capillary hyperpermeability produced by the intradermal injection of histamine. The fact that sodium salicylate was considerably less active in the adrenalectomized animal recalls the observation of Keleman (1957) that sodium salicylate was less active in the adrenalectomized rat than in the intact animal in antagonizing the oedema of the rat foot in response to an injection of 5-hydroxytryptamine.

It is noteworthy that cortisone acetate was active in preventing the capillary hyperpermeability reaction, since this is in contrast to its ineffectiveness against the formaldehyde reaction. The two aryloxypropionates tested here were inactive in a dose which afforded considerable protection against the effects of formaldehyde.

Anti-inflammatory agents in the adrenalectomized mouse. In the present experiments three anti-inflammatory agents proved to be less effective in the adrenalectomized animal than in its intact counterpart. This could be interpreted as meaning that all these agents stimulate the secretion of adrenal hormones which are, in fact, the active inflammatory agents. However, since cortisone and adrenaline were inactive in our test against formaldehyde-induced inflammation, this seems unlikely. It is more likely that the adrenal hormones play the part which Ingle (1952) has termed the " permissive " role of the adrenals. On this basis it is convenient to think of the adrenal secretion as acting upon the capillaries to render them sufficiently responsive to anti-inflammatory agents for the latter compounds to show their inherent activity. In the absence of the adrenal secretion, however, the capillaries are rendered refractory to any attempt to protect them from chemical damage.

DISCUSSION

Many compounds have been shown to cause an increased permeability of the capillaries when introduced into the tissues. These compounds may be classified into those which act upon the capillary endothelium to render it more permeable to water and plasma protein, and those which are more or less indiscriminately injurious to cells and release from such damaged cells substances which bring about capillary hyperpermeability. There can be little doubt that histamine belongs to the former category and has a direct action upon the capillary endothelium, rendering it more permeable than normal to both water and plasma proteins. There is also reason to suppose that formaldehyde, along with many other " irritant " substances, causes the liberation or activation of some substance or substances within the tissues, which in their turn act upon the capillaries to render them more permeable. It is not possible to say with any certainty what is the nature of the mediator or mediators which are involved in formaldehyde-induced inflammation, but the pharmacological evidence is already interesting and suggestive, and may eventually give us an answer to the problem.

Ungar (1952) has shown that it is possible to explain the anti-inflammatory action of the antipyretic-analgesic class of drugs on the basis of their anti-fibrinolysin activity, and it could be postulated that the ability of the various analgesic-antipyretic drugs capable of inhibiting formaldehyde-induced inflammation in the mouse foot

is due to their anti-fibrinolysin action. Recently, however, Collier, Holgate, Schachter & Shorley (1960) have shown that salicylate is capable of completely inhibiting the action of bradykinin on bronchial smooth muscle *in vivo*. Since this peptide also causes an increase in capillary permeability, it is possible that the inhibition of formaldehyde-induced inflammation which we have observed is due to an inhibition of the action of bradykinin released by the damaged tissues. Spector & Willoughby (1959) have investigated the increased permeability of the small blood vessels in response to turpentine, and have shown that it is due to the activation of a serum globulin system. This was inhibited by salicylate *in vitro*, as also was the increased permeability of the blood vessels *in vivo*. Besides these proposed direct actions of the analgesic-antipyretic drugs in antagonizing the production of inflammation, many workers have suggested that the salicylates, since they stimulate the pituitary-adrenal system, have an anti-inflammatory action due to released adrenocortical steroids. The evidence for this assertion has been critically reviewed by Smith (1953), who came to the conclusion that this mechanism, whilst it no doubt occurs, is not sufficient to explain all the anti-inflammatory actions of salicylates, notably their established effectiveness in rheumatoid diseases. It is probable that the analgesic-antipyretic class of drugs exert their effect by more than one mechanism.

We have shown that although salicylate is capable of antagonizing the inflammation produced by both histamine and formaldehyde, cortisone is only effective against histamine and the four aryloxypropionates tested are only effective against formaldehyde. Consequently any explanation of the mode of action of these inflammatory agents will have to take into account the selective effect of some antagonists. The inactivity of cortisone is in accord with its inactivity against thermal inflammation reported by Sevitt (1957), and its inactivity to the inflammation resulting from various bacterial toxins, which was shown by Lattes, Blunt, Rose, Jessor, Vaillencourt & Ragan (1953). Cortisone is also without any action against an ultra-violet radiation-induced erythema in guinea-pigs (Adams, personal communication). At present it is impossible to predict which substances, in the broad category of analgesic-antipyretics, will be active as antagonists to formaldehyde-induced inflammation.

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REFERENCES

BOURNE, G. H. (1951). Some histological aspects of formalin " arthritis " in rats. *Brit. J. exp. Path.*, **32**, 377–381.

BUCH, O. (1959). Anti-inflammatory properties of 3-phen. !propylcarbamate. *Arch. int. Pharmacodyn.*, **123**, 140–147.

COLLIER, H. O. J., HOLGATE, J. A., SCHACHTER, M. & SHORLEY, P. G. (1960). The bronchoconstrictor action of bradykinin in the guinea-pig. *Brit. J. Pharmacol.*, **15**, 290–297.

GESCHICKTER, C. F., O'MALLEY, W. E. & RUBACKY, E. P. (1960). A hypersensitivity phenomenon produced by stress: the " negative phase " reaction. *Amer. J. clin. Path.*, **34**, 1–4.

INGLE, D. J. (1952). Role of adrenal cortex in homeostasis. *J. Endocr.*, **8**, xxiii–xxvii.

KELEMAN, E. (1957). The inhibition by sodium salicylate of oedema of the hind-paw of the rat, induced by 5-hydroxytryptamine and hyaluronidase. *Brit. J. Pharmacol.*, **12**, 28–29.

LATTES, R., BLUNT, J. W., ROSE, H. M., JESSOR, R. A., VAILLENCOURT, DE G. & RAGAN, C. (1953). Lack of cortisone effect in the early stages of inflammation and repair. *Amer. J. Path.*, **29**, 1–19.

LOCKETT, M. F. & JARMAN, D. A. (1958). Assay of capillary action of flavanoid compounds in mice. *Brit. J. Pharmacol.*, **13**, 11–16.

PARRATT, J. R. & WEST, G. B. (1957). 5-Hydroxytryptamine and the anaphylactoid reaction in the rat. *J. Physiol.*, **139**, 27–41.

PARRATT, J. R. & WEST, G. B. (1958). Inhibition by various substances of oedema formation in the hind-paw of the rat induced by 5-hydroxytryptamine, histamine, eggwhite, and compound 48/80. *Brit. J. Pharmacol.*, **13**, 65–70.

SELYE, H. (1949). Further studies in the participation of the adrenal cortex in the pathogenesis of arthritis. *Brit. med. J.*, **2**, 1129–1135.

SETNIKAR, I., SALVATERRA, M. & TEMELCOU, O. (1959). Antiphlogistic activity of iproniazid. *Brit. J. Pharmacol.*, **14**, 484–487.

SEVITT, S. (1957). *Burns, Pathology and Therapeutic Implications.* London: Butterworth.

SMITH, M. J. H. (1953). Some recent advances in the pharmacology of salicylates. *J. Pharm. (Lond.)*, **5**, 81–93.

SMITH, M. J. H. (1955). The effects of sodium salicylate on blood glucose in the rat. *Brit. J. Pharmacol.*, **10**, 110–112.

SPECTOR, W. G. & WILLOUGHBY, D. A. (1959). The demonstration of the role of mediators in turpentine pleurisy in rats by experimental suppression of the inflammatory changes. *J. Path. Bact.*, **77**, 1–17.

SPECTOR, W. G. & WILLOUGHBY, D. A. (1960). The suppression by anti-esterases of increased capillary permeability in acute inflammation. *J. Path. Bact.*, **79**, 21–35.

UNGAR, G. (1952). Inflammation and its control—a biochemical approach. *Lancet*, **ii**, 742–745.

Brit. J. Pharmacol. (1961), **16**, 170–179.

SOME BIOLOGICAL PROPERTIES OF CEPHALOSPORIN C AND A DERIVATIVE

BY

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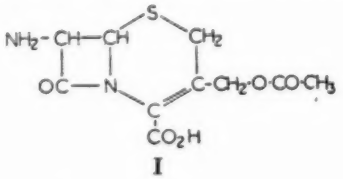
Some biological properties of cephalosporin C and of a pyridinium derivative, "cephalosporin C_A (pyridine)," were examined. Staphylococci, both penicillinase-producing and non-penicillinase-producing, and some other bacteria tested, were inhibited by 60 to 125 μg cephalosporin C/ml., and 5 to 20 μg cephalosporin C_A (pyridine)/ml. The ratio of the activity of the two antibiotics varied for different organisms. Resistance developed slowly on repeated subculture of penicillinase-producing staphylococci in presence of either antibiotic. The minimum inhibitory concentration of cephalosporin C_A (pyridine) upon penicillinase-producing staphylococci increased 4 to 8-fold with a 500-fold increase in inoculum size; with cephalosporin C there was a 2-fold increase. Their activity was not reduced by serum. Both substances were non-toxic. They were excreted quantitatively in the urine when given intravenously or subcutaneously to mice. After oral administration less than 5% of the dose was excreted. Cephalosporin C_A (pyridine) was about 8 times more active than cephalosporin C in protecting mice from an experimental streptococcal infection, nine doses of 6.25 mg/kg affording complete protection.

Cephalosporin C is an antibiotic which differs from the true penicillins in having as nucleus 7-aminocephalosporanic acid (I) (Abraham & Newton, 1961; Loder, Newton & Abraham, 1961) in place of 6-aminopenicillanic acid (II) (Sheehan, Henery-Logan & Johnson, 1953; Batchelor, Doyle, Nayler & Rolinson, 1959), the nucleus of the true penicillins, including cephalosporin N or synnematin B (Newton & Abraham, 1954; Abraham, Olson, Newton, Schuurmans, Schenck, Hargie, Fisher & Fusari, 1955). Cephalosporins N and C both have a D-α-aminoadipoyl side-chain. In cephalosporin C_A (pyridine) the acetoxy group of I appears to have been replaced by a pyridinium group (Abraham & Newton, 1958).



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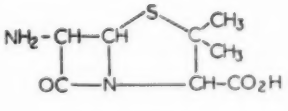

II

Cephalosporin C is much more resistant to penicillinase of *B. cereus* or *B. subtilis* than is benzylpenicillin (Abraham & Newton, 1956). It was decided to investigate the biological properties of cephalosporins C and C_A (pyridine), but the preliminary investigations reported here have been limited by lack of material.

METHODS

Antibiotic preparations. Cephalosporin C was obtained from the Medical Research Council Antibiotic Research Station, Clevedon, and was substantially pure. The pyridine derivative of cephalosporin C was prepared by Drs. Abraham and Newton (Hale, Abraham & Newton, 1961). It has been named "cephalosporin C_A (pyridine)" by these workers and was about 60% pure. Cephalosporin N was approximately 23% pure. The penicillin used was crystalline benzylpenicillin sodium (Boots Pure Drug Co.). Solutions were made in water and sterilized where necessary by passage through a sintered glass filter. In all cases results are expressed *in terms of the pure substances.*

Assay. This was carried out by the hole-plate or cylinder-plate method, with *Staphylococcus aureus* NCTC 6571 as test organism. A preparation of cephalosporin C of known purity was used as standard, not only for C but for the C_A (pyridine); though we were fully aware of the dangers of attempting to assay one substance in terms of another, this was felt at the time to be the least unsatisfactory course to follow.

The minimum inhibitory concentration was determined by the tube dilution method using 0.5 ml. vol. of Difco heart infusion broth (unless otherwise stated) and two-fold dilutions throughout. Tubes were read for the presence of visible growth after 24 hr (unless otherwise stated) and thereafter daily for 6 days.

Effect of inoculum size. The minimum inhibitory concentration was determined using as inoculum two drops (0.04 ml.) of (a) a 1 in 40, and (b) a 1 in 20,000 dilution in broth of a 24 hr broth culture.

Effect of serum on antibiotic activity. Two methods were used: (a) Solutions of antibiotic in water and in 50% horse serum were incubated at 37° C for 3 hr, then assayed by the plate method. (b) Two-fold dilutions of antibiotic in broth (containing 10% serum in the case of streptococci) and in broth containing 50% horse serum were inoculated with *Staphylococcus aureus* (strain R1) or *Streptococcus pyogenes* (CN10) and the minimum inhibitory concentration read after 24 hr incubation at 37°.

Acquisition of resistance. From the highest concentration showing growth in a dilution series, a transfer was made after 24 or 48 hr to a further range of tubes. This was repeated fourteen times, the end point being noted each time. The resistant organisms were then subcultured thirteen times in antibiotic-free broth and the minimum inhibitory concentration determined at the 5th, 9th, and 13th transfers.

Qualitative test for production by staphylococci of a penicillinase or of enzymes destroying cephalosporins C or C_A (pyridine). Equal volumes of antibiotic solution and 24 hr broth culture of the organism were incubated together for 1 hr at 37° C. The mixture was then assayed by the cylinder-plate method, using as controls (a) a solution of antibiotic in broth, and (b) antibiotic solution incubated for 1 hr with the non-penicillinase-producing *Staphylococcus aureus* NCTC 6571. Absence of inhibition zones round the cylinders containing test solution and their presence round those containing the controls was taken to indicate destruction of the antibiotic.

Serum concentration and rate of excretion in mice. The mice were catheterized and restrained as described by Heatley (1959) and antibiotic was given by various routes. Urine was assayed by the cylinder-plate method and serum by a vertical diffusion method (Heatley & Florey, 1953). *Staphylococcus aureus* NCTC 6571 was test organism for both assays. Standards were almost always prepared from a sample of the actual solution given to the animals, and for the serum assays they were made up in serum. Each urine sample was assayed in quadruplicate, but for serum often only single assays could be done, so that these values are only semi-quantitative. None of the animals was starved. Those given the antibiotic by the oral route were killed by coal gas after 2 or 4 hr. The stomach and small intestine were rinsed slowly with M/50 phosphate buffer *p*H 6.8, and the caecum and large intestine were likewise extracted; when the stomach or caecum could not easily be washed out, they were minced with scissors and extracted with the appropriate gut washings. These extracts were centrifuged and the supernatants assayed.

TABLE 1

COMPARATIVE ANTIBACTERIAL ACTIVITY OF BENZYLPENICILLIN AND OF CEPHALOSPORINS C, C_A (PYRIDINE), AND N

The inoculum was 2 drops of an overnight broth culture of the organism, diluted as indicated, per 0·5 ml. of medium, unless otherwise stated. The inoculum for *Myco. tuberculosis* was 0·1 ml. of a 6-day culture in Dubos medium, per 5 ml. HB=heart infusion broth; S=serum. The activity ratio C_A/C in the last column was calculated by dividing the min. inhibitory concentration of cephalosporin C by the min. inhibitory concentration of cephalosporin C_A (pyridine)

Organism	Inoculum broth culture diluted 1 in	Medium	Min. inhibitory concentration (μg/ml.) after 24 hr growth				Approx. ratio of activity
			C	C_A	N	Penicillin	C_A/C
Strep. pyogenes, CN10	40	HB+10% S	31	2·1	1·2	<0·1	15
Strep. viridans, NCTC 3165	40	HB+10% S	125	62·5	2·3	0·2	2
Pneumococcus, type I, CN33	40	HB+10% S	31	2·1	2·3	0·2	15
B. anthracis, avirulent	4,000	HB	31	7·8	1·1	0·1	4
Cl. welchii, NCTC 6125	Undil.	HB+5% S	125	40	1·1	0·2	3·1
Cl. septique	Undil.	HB+5% S	7·8	0·6	1·1	0·2	13
Cl. tetani, NCTC 279	Undil.	HB+5% S	3·9	1·2	1·1	<0·1	3·2
C. diphtheriae, gravis	4,000	HB	15·6	7·8	1·1	0·2	2
N. meningitidis	Undil.	HB+10% S	3·9	1·0	1·1	<0·1	4
N. gonorrhoeae							
NCTC 8676	Undil.	HB+10% S	7·8	1·0		—	8
NCTC 8375	Undil.	HB+10% S	1·9	1·0	1·1	0·2	2
NCTC 7129	Undil.	HB+10% S	1·9	2·0	—	—	1
Freshly isolated 1	Undil.	HB+10% S	3·9	1·0	—	—	4
Freshly isolated 2	Undil.	HB+10% S	15·6	2·5	—	0·1	6
Freshly isolated 3	Undil.	HB+10% S	15·6	1·2	—	0·1	13
Salm. typhi							
Rough	4,000	HB	15·6	15·6	2·3	4·8	1
Smooth	4,000	HB	15·6	27·4	1·1	—	1·8
Bact. friedländeri							
NCTC 5054	4,000	HB	31	31	4·6	19·2	1
Sh. shigae	4,000	HB	125	7·8	9·3	>6	16
Sh. sonnei	4,000	HB	125	7·8	—	>6	16
Escherichia coli, Type I							
Smooth	4,000	HB	125	13·7	9·3	>6	9
Rough	4,000	HB	125	6·9		—	18
Proteus vulgaris							
Non-spreading	4,000	HB	62·5	125	4·6	>6	0·5
Spreading	4,000	HB	31	110		—	0·3
Brucella abortus	40	HB+10% S	31	15·6	1·2	>6	2
Br. melitensis	40	HB+10% S	15·6	7·8		—	2
Haemophilus pertussis (incubated 2 days)	Undil.	Wheeler's	31	31	1·2	0·75	1
H. influenzae (incubated 2 days)	Undil.	Fildes'	31	31	9·3	6	1
Pseudomonas pyocyanea	4,000	HB	>1,000	>500	—	—	—
Vibrio cholerae							
Laboratory strain	4,000	HB	0·8	15	—	—	0·05
Strain 188	4,000	HB	2·0	13·7	—	—	0·14
Strain 189	4,000	HB	1·0	13·7	—	—	0·7
Mycobacterium tuberculosis H37Rv (human) (incubated 6 days)	Undil.	Dubos'	>200	>100	>9	>6	—
Branch (bovine) (incubated 6 days)	Undil.	Dubos'	>200	>100	>9	>6	—
Actinobovis israeli	Undil.	HB	>1,000	>500	>9	6	—
Cryptococcus neoformans	Undil.	Sabouraud's	>1,000	440		—	>0·5

RESULTS

Antibacterial properties against various organisms. Twenty-five different organisms were tested in parallel for sensitivity to cephalosporins C and C_A (pyridine). Table 1 gives the minimum inhibitory concentration after 24 hr incubation. After a further 5 days' incubation it had increased 2-fold for cephalosporin C and 2- to 4-fold for cephalosporin C_A (pyridine). The minimum inhibitory concentration for cephalosporin N and for penicillin, as determined by Heatley & Florey (1953), but quoted here in terms of pure substance, are also given for comparison.

Antibacterial activity against Staphylococcus aureus. Table 2 gives the min. inhibitory concentration after 24 hr incubation of cephalosporins C, C_A (pyridine) and N and also benzylpenicillin against 10 penicillinase-producing and 7 non-penicillinase-producing strains of *Staphylococcus aureus.* After 6 days the titre had decreased 2-fold for C and 4-fold for C_A (pyridine). That of penicillin had decreased about 2-fold for the non-penicillinase-producing strains and at least 8- to 64-fold for the penicillinase-producing strains.

Tables 1 and 2 show that *Staphylococcus aureus* is amongst the organisms more sensitive to cephalosporins C and C_A (pyridine) and, as with most other Gram-positive organisms, the latter is 6 to 30 times as effective as the former. But about twice as much C_A (pyridine) is needed to inhibit growth of the penicillinase-producing as of the non-penicillinase-producing strains. This was confirmed with 36 further strains of *Staphylococcus aureus*; 18 were penicillinase- and 18 non-penicillinase-producers, their growth being inhibited by 13.6 and 6.8 μg C_A (pyridine)/ml. respectively on agar ditch plates.

TABLE 2

COMPARATIVE ACTIVITY OF CEPHALOSPORINS C, N, AND C_A (PYRIDINE) AND BENZYLPENICILLIN AGAINST *STAPHYLOCOCCUS AUREUS*

Two-fold dilutions (0·5 ml.) in heart broth were inoculated with 0·04 ml. of a 1 in 40 dilution of 24-hr broth culture. Tubes were read after 24 hr incubation. All R and RD strains had been recently isolated from patients

	Min. inhibitory concentration (μg/ml.)					Ratio of activity				
Strain	C	C_A	Pen.	N	C_A/C	Pen/C	N/C	Pen/C_A	N/C_A	Pen/N
Penicillinase-producing strains										
G2	125	5·2	500	230	24	0·25	0·54	0·01	0·023	0·46
R1	125	7·8	500	230	16	0·25	0·54	0·016	0·034	0·46
R2	62	7·8	500	230	8	0·125	0·27	0·016	0·034	0·46
R3	62	7·8	500	230	8	0·125	0·27	0·016	0·034	0·46
R4	125	15·6	500	230	8	0·25	0·54	0·03	0·068	0·46
R5	125	7·8	500	230	16	0·25	0·54	0·016	0·034	0·46
R6	125	7·8	500	230	16	0·25	0·54	0·016	0·034	0·46
D3R	125	3·9	31	29	32	4	4·3	0·12	0·13	0·93
Carol	100	16·7	125	230	6	0·8	0·43	0·13	0·073	1·8
Bartlett	100	16·7	250	120	6	0·4	0·83	0·067	0·14	0·48
Non-penicillinase-producing strains										
RD21	62	4·9	0·03	3·6	13	2,100	17	160	1·4	120
RD23	62	4·9	0·03	1·8	13	2,100	35	160	2·7	60
RD24	125	19·8	0·06	1·8	6·3	2,100	70	330	11	30
RD25	62	4·9	0·03	1·8	13	2,100	35	160	2·7	60
RD26	62	4·9	0·03	1·8	13	2,100	35	160	2·7	60
RD27	62	4·9	0·01	1·8	13	6,200	35	490	2·7	180
NCTC 6571	125	4·2	0·015	1·	30	8,300	70	280	2·3	120

TABLE 3

DEVELOPMENT OF RESISTANCE TO CEPHALOSPORINS C AND C_A (PYRIDINE) IN PENICILLINASE-PRODUCING STRAINS OF *STAPHYLOCOCCUS AUREUS*

Figures represent -fold increase of min. inhibitory concentration over original

Strain	Subculture no. in presence of antibiotic														In plain broth		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	5	9	13
	Cephalosporin C_A (pyridine)																
R1	4	8	8	4	16	16	16	16	16	16	32	32	32	32	8	4	4
R2	8	8	4	8	16	16	8	8	8	16	32	16	32	32	8	8	4
R3	4	16	8	16	16	16	8	8	32	32	32	32	32	32	4	4	4
R4	4	8	8	8	16	16	8	8	8	16	32	16	32	32	8	4	2
R5	4	8	4	8	16	16	8	8	8	16	32	16	32	32	4	4	4
R6	4	8	8	16	16	32	16	16	16	16	32	32	16	32	16	16	16
D3R	2	2	8	8	16	16	32	64	32	32	32	64	32	64	—	—	32
B7	2	8	4	8	16	16	16	8	8	16	32	16	32	64	8	4	4
B8	4	8	8	16	16	16	16	8	16	16	32	32	32	64	8	4	4
B9	2	4	4	4	4	8	8	8	4	8	16	8	8	8	4	4	4
B10	8	16	16	16	16	32	16	32	16	16	64	64	32	64	16	8	8
B11	4	16	8	16	32	32	16	16	16	32	64	32	32	32	8	8	4
B12	4	8	8	8	16	32	16	16	16	32	32	32	32	32	16	16	16
	Cephalosporin C																
R1	0	0	4	2	8	4	4	8	4	8	4	8	8	8	—	—	2
D3R	2	2	4	2	8	8	8	16	8	8	8	8	16	16	—	—	16

Effect of inoculum size. The effect on min. inhibitory concentration of cephalosporins C and C_A (pyridine) and of penicillin of a 500-fold change in inoculum size was tested against 10 penicillinase-producing *Staphylococcus aureus* strains, and against the non-penicillinase-producing *Staphylococcus aureus* NCTC 6571. The heavier inoculum caused an average increase of 2-fold for cephalosporin C and of 4-fold for C_A (pyridine); for penicillin there was no change for NCTC 6571, but an increase of 256 to 2,000-fold for the penicillinase-producing strains.

The decrease in titre which took place when the incubation was prolonged for 3 days was slightly greater for C_A (pyridine) and penicillin when a large, than when a small, inoculum had been used. For cephalosporin C there was no difference.

Effect of serum. The activities of cephalosporins C and C_A (pyridine) were not appreciably altered by the presence of 50% horse serum in either solid or liquid medium.

Induction of resistance in penicillinase-producing strains of Staphylococcus aureus. Table 3 summarizes the changes in titre when 13 penicillinase-producing strains of *Staphylococcus aureus* were made resistant to cephalosporin C_A (pyridine) and 2 strains to cephalosporin C, and during their subsequent repeated subculture in antibiotic-free medium.

The resistant organisms, which grew somewhat more slowly than the sensitive parent strains, were unstable on repeated subculture in antibiotic-free medium, although no strain had reverted to its original sensitivity after 13 such transfers. Resistance to C_A (pyridine) developed more readily than to cephalosporin C.

Cross resistance. Six of the thirteen strains made resistant to cephalosporin C_A (pyridine) were tested for penicillin sensitivity. It was found not to have changed. Only two strains, R1 and D3R, were studied for cross resistance between cephalosporins C and C_A (pyridine). Whereas organisms made resistant to cephalosporin C became almost equally resistant to cephalosporin C_A (pyridine), those made resistant to cephalosporin C_A (pyridine) showed a relatively smaller increase to cephalosporin C. Clearly cross resistance between the antibiotics develops, but the pattern shown by the strains studied may not be typical.

Penicillinase and cephalosporinase activity of Staphylococcus aureus. By the qualitative method used 7 of the 10 penicillin-resistant strains were shown to be powerful penicillinase producers. Two strains (Carol & Bartlett) were less active and strain D3R was relatively feeble. There was scarcely detectable destruction of 1 mg or 0.5 mg/ml. of cephalosporins C or C_A (pyridine) by any of the strains, though the method was not sensitive enough to detect weak enzyme action with certainty.

Acute toxicity. The acute toxicity of cephalosporin C has already been briefly reported by Florey (1955, 1956), who found that a dose of 100 mg of material now known to be about 75% pure had no effect when injected intravenously into a 20 g mouse.

Solutions of cephalosporin C_A (pyridine) in 0.45 ml. distilled water were given intravenously to 20 g mice. Three mice were unaffected by 30 mg of 67% pure material and a fourth by 50 mg of the same product (equivalent to 1,000 and 1,670

mg of pure cephalosporin C_A (pyridine)/kg). A fifth mouse died at once when 100 mg of a different preparation, 50% pure, was injected slowly (2,500 mg of pure C_A (pyridine)/kg). No macroscopic or microscopic lesions were found in liver, spleen or kidneys of any of these mice.

Local toxicity. The injection of 0.1 ml. of a 10% solution of cephalosporin C_A (pyridine) in saline subcutaneously into 3 mice, and of 0.05 ml. intramuscularly into another 3 mice, caused no local reaction visible at post-mortem examination 5 days later, apart from a pin-point area of pigmentation at the site of the subcutaneous injection.

Serum concentration and excretion in mice. The limited number of experiments and the great scatter between replicates enables only tentative conclusions to be drawn. Table 4 shows that after intravenous or subcutaneous injection both cephalosporins C and C_A (pyridine) are excreted rapidly in the urine, largely within the first 2 hr. In this respect they resemble benzylpenicillin, but whereas total urinary

TABLE 4

SERUM CONCENTRATION AND URINARY EXCRETION OF CEPHALOSPORINS C AND C_A (PYRIDINE) AFTER INTRAVENOUS AND SUBCUTANEOUS ADMINISTRATION TO 20 G MICE

	Serum concn. (μg/ml.) after			% of dose excreted in urine in period (hr) after dose				
Route	1 hr	2 hr	3 hr	0–1	1–2	2–4	4–6	Total
Cephalosporin C (dose, 250 mg/kg)								
Intraven.	—	—	—	93		<6·3	<3·8	<93
Intraven.	50	—	—	91		<8·2	<4·7	<91
Intraven.	27	<20	<20	130		<7·5	<4·7	<130
Subcut.	140	—	—	70·2	38·1	<4·3	<1·1	<108
Subcut.	—	—	—	88·5	32·8	<4·3	<1·1	<121
Subcut.	190	42	11	62·8	37·2	<4·0		<100
Subcut.	145	27	<12	33·1	27·6	23·8	6·3	91
Cephalosporin C_A (pyridine) (dose, 200 mg/kg)								
Intraven.	80	<16	—	111		4·7	0·2	116
Intraven.	43	<16	—	135		1·2	0·2	136
Intraven.	69	<16	—	—		16·0	0·2	—
Intraven.	50	<14	—	99		8·8	<2·5	<108
Intraven.	41	<12	—	99·5		4·5	<2·6	<104
Subcut.	—	—	—	98·3		6·1	1·1	106
	—	<6	—	—		—	—	—
Benzylpenicillin (dose, 200 mg/kg)								
Intraven.	164	39	11	7·7	10·5	4·3	2·4	24·9
Intraven.	62	11	3·1	49·0	6·0	1·0	0·1	56·1
Intraven.	44	2	4	68·6	1·2	0·1	0	69·9
Intraven.	64	6	1	35·6	4·0	0·5	0·1	40·2
Intraven.	95	9	—	95·4	7·8	0·5	—	103·7
Intraven.	260	84	—	42·1	14·9	0·5	—	57·5
Intraven.	125	6	—	80·6	5·2	0·1	—	85·9
Intraven.	105	6·4	4	47·0	13·6	1·6	0·1	62·3
Subcut.	—	—	—	34·0	21·2	6·4	0·4	62·0
Subcut.	52·5	4·2	0·7	10·8	11·9	2·3	0·02	25·0
Subcut.	400	16	2·7	40·0	24·0	2·5	0·2	66·7

excretion of the latter averaged 60% of the dose, the whole of the cephalosporins appeared in the urine—indeed the urinary output was rather more than 100% of the administered dose.

After oral administration (see Fig. 1) the urinary excretion of both substances averaged less than 5% of the dose and was much the same whether the mouse was killed after 2 or 4 hr. This, together with the fact that the stomach and small intestine contained much less antibiotic at 4 than at 2 hr whereas the caecum and

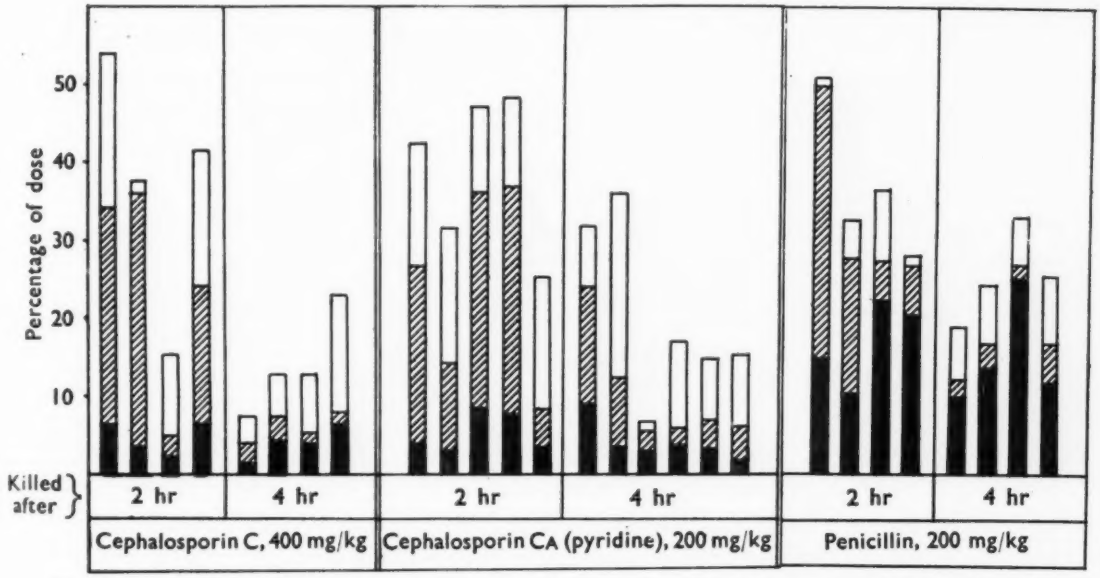

Fig. 1. Percentage of oral dose of various antibiotics given to mice, recovered 2 or 4 hr after administration; in urine ■; from stomach+small intestine ▨; from caecum+large intestine □.

large intestine contained about the same amount, suggests that little absorption takes place from the lower parts of the gut. That considerable destruction takes place is shown by the percentage of dose unaccounted for:

	Percentage of dose unaccounted for after	
	2 hr	4 hr
Cephalosporin C	46–85	77–93
Cephalosporin C_A (pyridine)	52–75	64–93
(Benzylpenicillin	49–72	67–81)

Since both substances appear to be quantitatively excreted after parenteral administration, it follows that no detectable destruction takes place in the body tissues; such loss as there is presumably occurs in the gut. Absorption from the gut is slight.

Mouse protection experiments. Florey (1955, 1956) reported that eight 3-hourly doses of 1 mg of cephalosporin C completely protected, 0.5 mg and 0.25 mg prolonged life and 0.125 mg had no effect in mice infected intraperitoneally with *Streptococcus pyogenes* (CN10) which produced 100% mortality of controls in 12 hr.

The experiment was repeated using cephalosporin C_A (pyridine). The results (Table 5, 1) show that 9 doses of 0.125 mg (6.25 mg/kg) or more completely protected the mice. Table 5, 2, shows the results of a milder infection with the same organism which gave 80% mortality of the controls after 2 weeks. Nine doses of 0.0625 mg (3.12 mg/kg) or more completely protected, and of 0.0312 mg (1.6 mg/kg) partially protected, the mice. In both experiments all surviving mice gave negative blood cultures.

TABLE 5

SURVIVAL OF MICE TREATED WITH CEPHALOSPORIN C_A (PYRIDINE) AFTER INFECTION WITH *STREP. PYOGENES*

Mice were infected intraperitoneally with 0·5 ml. of a 1 in 10 dilution of 24-hr broth culture of *Streptococcus pyogenes* CN10. The test groups received 9 doses subcutaneously of cephalosporin C_A (pyridine) in 0·3 ml. saline every 3 hr, starting 1 hr after infection

Expt. no.	Time after infection	Dose (mg)					
		0·5	0·25	0·125	0·0625	0·031	0 (control)
1	25 hr	5/5	5/5	5/5	4/5	1/5	0/25
	4 days	5/5	5/5	5/5	0/5	1/5	0/25
	4 weeks	5/5	5/5	5/5	0/5	0/5	0/25
2	24 hr	5/5	5/5	5/5	5/5	5/5	13/25
	2 weeks	5/5	5/5	5/5	5/5	4/5	5/25
	3 weeks	5/5	5/5	5/5	5/5	3/5	5/25

DISCUSSION

The mode of action of cephalosporins C and C_A (pyridine) upon bacteria is essentially the same as that of cephalosporin N and the common penicillins, all being powerfully bactericidal and bringing about the lysis of growing bacteria (Crawford, Loder, Abrahams & Newton, as quoted by Abraham & Newton, 1958). Comparisons of activities based on 2-fold dilution tests may have an error of up to 4-fold ; for this reason, and because relatively few strains have been examined, generalizations about antibacterial range, etc., must be only tentative. Cephalosporin C_A (pyridine) was 2 to 32 times more active than cephalosporin C against most Gram-positive organisms, including all staphylococci tested, although against several Gram-negative organisms there was little difference ; against shigellae and *Escherichia coli* it was slightly more active, but against three strains of *Vibrio cholerae* cephalosporin C was 7 to 18 times more active than cephalosporin C_A (pyridine) and more active than either benzylpenicillin or cephalosporin N. Although the penicillin was the most active of the four antibiotics against certain Gram-positive pathogens and Neisseria, cephalosporins C and C_A (pyridine) were both more active against the penicillinase-producing staphylococci ; furthermore, the activity of cephalosporin C was virtually independent of inoculum size and that of cephalosporin C_A (pyridine) almost so.

On repeated subculture in medium containing antibiotic, penicillinase-producing staphylococci gradually became resistant to cephalosporins C and C_A (pyridine)—somewhat more easily to the latter—but this resistance was gradually lost on cultivation in the absence of antibiotic. There was partial cross resistance between cephalosporins C and C_A (pyridine) but none to benzylpenicillin in the few strains tested.

Like cephalosporin C, cephalosporin C_A (pyridine) was not inactivated by serum, nor had it appreciable toxicity. It appeared to be about 8 times as effective as cephalosporin C in protecting mice from experimental streptococcal infection.

Both substances seemed to be excreted quantitatively in the urine after intravenous or subcutaneous administration to mice, though less than 5% of an oral dose was absorbed into the blood stream in spite of their acid stability; for the acid-labile benzylpenicillin the urinary recoveries in mice were 60% after intravenous or subcutaneous administration, and 16% after an oral dose. The amount of an oral dose unaccounted for was much the same for penicillin and the two cephalosporins in spite of the greater stability of the latter to acid and to penicillinase. The poor absorption from the gut and the high urinary excretion of cephalosporins C and C_A (pyridine) may well be a consequence of the polar nature of the side-chain, rather than a property associated with the nucleus.

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REFERENCES

ABRAHAM, E. P. & NEWTON, G. G. F. (1956). A comparison of the action of penicillinase on benzylpenicillin and cephalosporin N and the competitive inhibition of penicillinase by cephalosporin C. *Biochem. J.*, **63**, 628–634.

ABRAHAM, E. P. & NEWTON, G. G. F. (1958). Structure and function of some sulphur-containing peptides. *Ciba Foundation Symposium on Amino Acids and Peptides with Antimetabolic Activity*, p. 205. London: J. & A. Churchill Ltd.

ABRAHAM, E. P. & NEWTON, G. G. F. (1961). The structure of cephalosporin C. *Biochem. J.* In the press.

ABRAHAM, E. P., OLSON, B. H., NEWTON, G. G. F., SCHUURMANS, D. M., SCHENCK, J. R., HARGIE, M. P., FISHER, M. W. & FUSARI, S. A. (1955). Identity of cephalosporin *N* and synnematin *B*. *Nature, Lond.*, **176**, 551.

BATCHELOR, F. R., DOYLE, F. P., NAYLER, J. H. C. & ROLINSON, G. N. (1959). Synthesis of penicillin: 6-aminopenicillanic acid in penicillin fermentations. *Nature (Lond.)*, **183**, 257–258.

FLOREY, H. W. (1955). Antibiotic products of a versatile fungus. *Ann. intern. Med.*, **43**, 480–490.

FLOREY, H. W. (1956). The medical aspects of the development of resistance to antibiotics. *G. Microbiol.*, **2**, 361–370.

HALE, C. W., ABRAHAM, E. P. & NEWTON, G. G. F. (1961). Derivatives of cephalosporin C with certain heterocyclic tertiary bases. The cephalosporin C_A bases. *Biochem. J.* In the press.

HEATLEY, N. G. (1959). An arrangement for obtaining successive urine and blood samples from the unanaesthetized mouse. *Quart. J. exp. Physiol.*, **44**, 371–376.

HEATLEY, N. G. & FLOREY, H. W. (1953). A comparison of the biological properties of cephalosporin N and penicillin. *Brit. J. Pharmacol.*, **8**, 252–258.

LODER, B., NEWTON, G. G. F. & ABRAHAM, E. P. (1961). The cephalosporin C nucleus (7-aminocephalosporanic acid) and some of its derivatives. *Biochem. J.* In the press.

NEWTON, G. G. F. & ABRAHAM, E. P. (1954). Degradation, structure and some derivatives of cephalosporin N. *Biochem. J.*, **58**, 103–111.

SHEEHAN, J. C., HENERY-LOGAN, K. R. & JOHNSON, D. A. (1953). The synthesis of substituted penicillins and simpler structural analogs. VII. The cyclization of a penicilloate derivative to methyl phthalimidopenicillanate. *J. Amer. chem. Soc.*, **75**, 3292–3293.

Brit. J. Pharmacol. (1961), **16**, 180–187.

SALICYLATE AND LIVER GLUTATHIONE

BY

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The effects of sodium salicylate, benzoate, gentisate, m- and p-hydroxybenzoates, antipyrine, and 2:4-dinitrophenol on the reduced glutathione concentration of rat liver were determined. An increase in concentration in the male, but not in the female, rats was found after salicylate, benzoate and gentisate; after antipyrine, a comparable increase occurred in the females only. There is a relation, in these compounds, between capacity to increase the concentration of reduced glutathione in the rat liver in one sex and therapeutic activity in rheumatic fever.

In continuation of a search for pharmacological actions of salicylate which are correlated with its therapeutic properties, the effect of salicylate on the reduced glutathione concentration of liver was examined. A two-fold increase in the reduced glutathione concentration of rat liver after administration of sodium salicylate has been reported by Lutwak-Mann (1942). The present results do not confirm this finding: a moderate increase in the reduced glutathione concentration of rat liver was induced by salicylate, but in the male sex only.

An attempt has been made to evaluate this finding, by comparing the activity of salicylate on rat liver glutathione with that of a variety of other compounds. In addition to salicylate, the following were studied: other antirheumatic drugs of the same series (benzoate and gentisate), and of a different series (antipyrine); m- and p-hydroxybenzoates, which are therapeutically inert isomers of salicylate, and 2:4-dinitrophenol, which, like salicylate, is a peripheral metabolic stimulant.

In the first experiments, an interval between administration of the drug and termination of the experiment was arbitrarily selected as adequate and convenient for salicylate. This interval was unlikely to be suitable for the rapidly excreted compounds such as benzoate. Rather than find the optimal interval for each compound, a second series of observations was made, in which two doses were given of each of the compounds which in single doses of 200 mg/100 g body weight were tolerated without noticeable effect. Salicylate was included for comparative purposes.

A relation was then found, within the present group of compounds, between therapeutic activity in rheumatic fever and capacity to increase rat liver glutathione concentration in one sex.

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Since the quantity of oxidized glutathione extractable from rat liver is negligible (Bhattacharya, Robson & Stewart, 1955), the present investigation was confined to the reduced (GSH) form, which will be referred to as "glutathione."

METHODS

The drugs were given by intraperitoneal injection to young mature Wistar albino rats of 150–250 g body weight; with the exception of antipyrine, all the drugs were given as neutral solutions of the sodium salt.

Dosage. In the first series of experiments, each rat received a single dose of the drug, 17 hr before being killed; the doses were, in mg/100 g body weight: sodium gentisate, benzoate, m- and p-hydroxybenzoates, 100; sodium salicylate and antipyrine, 50; sodium 2:4-dinitrophenate, 2. In the second series, from which antipyrine and 2:4-DNP were omitted, two equal doses of each drug were given, 24 and 17 hr before the rats were killed; the doses were, in mg/100 g body weight: sodium gentisate, m- and p-hydroxybenzoates, 100; sodium benzoate, 75; sodium salicylate, 35.

General procedure. In both series of experiments, each drug was given to 14 rats, 7 of each sex. Each series included a control group of 28 rats, 14 of each sex; these were given 1 ml. 0.9% saline intraperitoneally when the corresponding treated animals were injected. A further control group of 14 was given no saline. The rats were put up daily in batches of 9 (first series) or 7 (second series) of the same sex; two rats of each batch were assigned to the control group, and one to each of the drugs; access to food (M.R.C. diet 41) and water was permitted throughout. Next day, the animals were killed between 9.00 and 9.10 a.m. by decapitation, the carcasses bled, and liver samples taken for the estimation of reduced glutathione.

Estimation of reduced glutathione. Reduced glutathione estimation was by a modification of the specific glyoxalase method (Woodward, 1935), where the course of the reaction was followed in terms of the residual methyl glyoxal, determined colorimetrically as its bis-2:4-dinitrophenylhydrazone.

The tissue extracts were prepared in 3% (w/v) sulphosalicylic acid (Bhattacharya, Robson & Stewart, 1955), using an all-glass Potter & Elvehjem (1936) type tissue grinder, with a pestle clearance of 0.002 to 0.004 in., operated at 8,000 rev/min. The glutathione standards were prepared from Reduced Glutathione (Sigma), m.p. 194.5° C. The glyoxalase reaction was conducted according to Schroeder & Woodward (1939), except that the initial concentration of methyl glyoxal in the reaction mixture was reduced from 1.0 to 0.75 mg/ml.

Estimation of the methyl glyoxal as its bis-2:4-dinitrophenylhydrazone depended on isolation of the precipitate by filtration. A filter stick was made for this purpose by connecting to a water pump a 7 in. length of 8.5 mm diameter × 6 mm bore glass tubing, the free end of which was cut squarely and lightly fire-polished. A glass bead of 6.0 to 6.5 mm diameter was sucked on to the end of this tube, which was then dipped into an aqueous suspension of short fibrous asbestos. The asbestos packed at the junction of the bead and tube, forming an efficient filter, which was washed by the passage of 20 to 30 ml. water.

The method finally adopted was as follows: The incubation of each glyoxalase reaction mixture was terminated by the addition of 10 ml. 0.025 N sulphuric acid; after spinning, 1 ml. of the supernatant was added to 2 ml. of 10% trichloroacetic acid, and 1 ml. of this mixture was added to 1 ml. of 2:4-dinitrophenylhydrazine reagent (made freshly every day by dissolving 120 mg 2:4-dinitrophenylhydrazine in 100 ml. of 2 N hydrochloric acid), and left standing for 18 hr. Using the filter stick, the supernatant was then removed, and the precipitate washed with 4 to 5 ml. water. Chloroform (10 ml.) was pipetted into the precipitation test-tube, the filter stick was disconnected from the water-pump, and the glass bead and asbestos discharged into the chloroform by gently blowing down the filter stick, the end of which was finally washed with a little water on to the chloroform. The precipitate was

dissolved in the chloroform, using a high-speed stirrer, and the bulk of the asbestos in the small aqueous layer was removed by suction tube and wash bottle. The solution of the hydrazone in chloroform was centrifuged briefly to ensure adequate separation of the aqueous layer, and a 1 ml. aliquot was added to a further 3 ml. chloroform.

The optical densities of the resulting solutions were measured at 435 mμ ; it was advantageous to set the spectrophotometer to an optical density of 0.350 for the zero glutathione standard. A curve, relating optical density directly to glutathione concentration, was drawn for each batch of analyses, and the unknown glutathione values were finally obtained by interpolation. The liver glutathione concentrations are expressed as mg/g wet weight.

Statistical. The two series of observations are treated separately. The variances and means found are compared, using the two-tailed F-test and the t-test (Snedecor, 1956). Comparable data, homogeneous in variance according to Bartlett's (1937) test, are pooled for purposes of estimation.

RESULTS

The precision of the estimation of liver glutathione was established by quadruplicate determinations in four rats: the estimated standard deviation of a single replicate was ±0.08 mg/g.

TABLE 1

THE CONCENTRATIONS OF REDUCED GLUTATHIONE IN THE LIVERS OF WISTAR ALBINO RATS

Intraperitoneal injections of the compounds were made 17 hr before.
M = male. F = female.

Drug	Dose mg/100 g body weight	Sex	No. of rats	Glutathione concentration mean ± s.d., mg/g
None	—	M	7	2·58 ± 0·44
		F	7	2·64 ± 0·24
0·9% NaCl, 1 ml.	—	M	14	2·48 ± 0·40
		F	14	2·55 ± 0·40
Sodium m-hydroxybenzoate	100	M	7	2·77 ± 0·56
		F	7	2·56 ± 0·32
Sodium p-hydroxybenzoate	100	M	7	2·79 ± 0·67
		F	7	2·64 ± 0·28
Sodium benzoate	100	M	7	2·75 ± 0·28
		F	7	2·71 ± 0·36
Sodium gentisate	100	M	7	2·47 ± 0·32
		F	7	2·64 ± 0·32
Sodium salicylate	50	M	7	3·63 ± 0·73
		F	7	2·51 ± 0·44
Antipyrine	50	M	7	2·31 ± 0·65
		F	7	3·32 ± 0·73
Sodium 2 : 4-dinitrophenate	2	M	7	2·49 ± 0·56
		F	7	2·58 ± 0·36

All drugs were tolerated in apparent comfort by the rats ; no distinctive signs were seen, except after antipyrine, which induced for some hours a mild stupor, from which the animals could be easily roused.

Single-dose experiments

The liver glutathione concentrations of the controls shown in Table 1 are homogeneous, and on pooling the data of the 14 untreated rats with those of the 28 rats given an injection of saline, the grand mean liver glutathione concentration of the controls of the first series is 2.55 mg/g, and the standard deviation (s.d.) ± 0.36.

A single dose of benzoate or gentisate did not affect rat liver glutathione concentration 17 hr later. The glutathione concentrations after the injection of these drugs are homogeneous, and these data, summarized in Table 1, when pooled, give a mean of 2.64 mg/g and s.d. ± 0.33, which are similar to the control values.

No distinction was found between the liver glutathione concentrations of the rats given m- or p-hydroxybenzoates. The female data resemble the controls, while the males show an elevated variance (see Table 1). On pooling the glutathione concentrations of each sex, the following estimates of mean and s.d. are obtained: male, 2.78 ± 0.60 mg/g; female, 2.60 ± 0.29 mg/g. The variance of the glutathione concentrations of the males is significantly greater than that of the controls, $0.05 > P > 0.01$.

After the single dose of salicylate or antipyrine, an increase in the mean liver glutathione concentration was found, but in one sex only (see Table 1). The mean glutathione concentration of the livers of the salicylate-treated males is 1.12 mg/g higher than the corresponding female figure, and this difference is significant, $0.01 > P > 0.001$. The mean concentration of glutathione in the livers of the

TABLE 2

THE CONCENTRATIONS OF REDUCED GLUTATHIONE IN THE LIVERS OF WISTAR ALBINO RATS

Two equal doses, shown in the second column, were given by intraperitoneal injection 24 and 17 hr previously. M = male. F = female.

Drug	Dose, mg/100 g body weight	Sex	No. of rats	Glutathione concentration mean ± s.d., mg/g
0·9% NaCl, 1 ml.	—	M	14	2·59 ± 0·17
		F	14	2·61 ± 0·22
Sodium m-hydroxybenzoate	100	M	7	2·69 ± 0·39
		F	7	3·08 ± 0·75
Sodium p-hydroxybenzoate	100	M	7	2·79 ± 0·48
		F	7	2·97 ± 0·60
Sodium benzoate	75	M	7	3·33 ± 0·30
		F	7	2·27 ± 0·75
Sodium gentisate	100	M	7	3·07 ± 0·33
		F	7	2·59 ± 0·41
Sodium salicylate	35	M	7	3·08 ± 0·39
		F	7	2·42 ± 0·36

antipyrine-treated females is 1.01 mg/g higher than that of the males, and this sex difference is also significant, $0.02 > P > 0.01$. With the exception of the salicylate-treated females, the variances of these groups are greater than that of the controls.

The single dose of sodium 2:4-dinitrophenate had no effect on the liver glutathione of the rat.

Two-dose experiments

The mean glutathione concentration in the livers of the 28 rats given two injections of saline, shown in Table 2, scarcely differs from the mean of those given one injection of saline. However, the variance of the control group of the second series of experiments is significantly less than that of the first, $P<0.01$.

The glutathione concentrations after 2 doses of m- or of p-hydroxybenzoate, shown in Table 2, are homogeneous in variance and on pooling yield a mean and s.d. of 2.88 ± 0.56 mg/g. The variance of the pooled data is significantly greater than that of the corresponding controls, $P<0.01$.

The glutathione concentrations, shown in Table 2, of livers from the rats given 2 doses of benzoate, gentisate, or salicylate are alike. In each case, the female mean is similar to that of the controls, while the male mean is substantially greater. Each of these sex differences in liver glutathione concentration is significant: $0.01>P>0.001$ (benzoate); $0.05>P>0.02$ (gentisate); $0.02>P>0.01$ (salicylate). The data for each sex are homogeneous, and, on pooling, the following estimates of mean and s.d. are obtained: male, 3.14 ± 0.35 mg/g; female, 2.43 ± 0.53 mg/g. The estimates of variance are significantly greater than that of the controls: male, $0.01>P>0.001$, female $P<0.001$.

DISCUSSION

The reduction in variance of the control glutathione concentrations after two injections of saline, as compared with one or none, suggests that the emotional disturbance of unaccustomed handling may affect liver glutathione; it is consistent with the increased turnover rate of glutathione in the liver of rats given adrenaline, observed by Henriques, Henriques & Mandelbaum (1957). The utility of the two-dose experiments is enhanced by this feature.

The increase in rat liver glutathione concentration produced by salicylate has been found, in the present experiments, not only to be smaller than that reported by Lutwak-Mann (1942), but also to be confined to the male sex. The apparent discrepancy between the present results and those of Lutwak-Mann (1942) may be attributable to differences in technique, since the method of estimating glutathione used by Lutwak-Mann was not specific (Patterson & Lazorow, 1955). The interpretation of the sex difference is a matter for further investigation: in particular, it would be desirable to determine whether the turnover rates of glutathione in the liver after salicylate are the same in both sexes.

The failure of a single dose of benzoate or gentisate to affect liver glutathione could be attributed to prompt excretion. In contrast, salicylate is excreted rather slowly in the rat, with a half-time of plasma concentration of 10 hr (Andrews, 1958).

When two doses were given, salicylate, benzoate and gentisate were indistinguishable in their effect on rat liver glutathione concentration; this effect was

not seen after the injection of the therapeutically inert m- and p-hydroxybenzoates, nor after 2:4-dinitrophenol. The effect of antipyrine on liver glutathione was comparable to that of salicylate, but the sex difference was reversed: elevation of liver glutathione concentration occurred only in the females. Although it is not possible, at present, to explain these results, it is worth considering whether the effect, particularly of salicylate, benzoate and gentisate, on liver glutathione concentration is related to some other property of these compounds.

The identity of the effects of two doses of salicylate, benzoate or gentisate on glutathione concentration in the rat liver cannot be accounted for in terms of conjugation, since glutathione is not the source of glycine in hippurate formation by the rat (Waelsch & Rittenberg, 1941). Exhaustion of the "first glycine pool" of Arnstein & Neuberger (1951) by massive doses of hippurate precursors might nevertheless affect liver glutathione concentration. The increased variance of liver glutathione concentration after m- and p-hydroxybenzoates could be accounted for in this way. However, the increases in mean glutathione concentration are not dependent on glycine conjugation, since this is improbable with antipyrine and gentisate, and since the rat forms only a negligible proportion of salicylurate (Quilley & Smith, 1952).

The calorigenic action of salicylate is not relevant to the present findings, because no sex difference in this effect was found in the rat by Andrews (1958), and because neither benzoate nor gentisate possesses this property (Meade, 1954; Andrews, 1958). Furthermore, 2:4-dinitrophenol, which is a powerful metabolic stimulant, had no effect on rat liver glutathione concentration.

The classical instance of increase in liver glutathione occurs in thyroid deficiency (Lazorow, 1954), and both salicylate (Austen, Rubini, Meroney & Wolff, 1958) and gentisate (Wolff & Austen, 1958) have definite antithyroid activity. However, 2:4-dinitrophenol is also a thyroid depressant (Wolff, Rubin & Chaikoff, 1950; Goldberg, Wolff & Greep, 1955), but did not affect liver glutathione, which eliminates this factor as an explanation of the present results.

Within the hydroxybenzoate series of compounds, therapeutic activity in rheumatic fever is not peculiar to salicylate. m- and p-Hydroxybenzoates are therapeutically inert, but benzoate has an unmistakable, if weak, anti-rheumatic action (Stockman, 1920). Gentisate, although active, is also inferior to salicyate (Horder & Bywaters, 1955). It is apparent, therefore, that in these compounds there is an association between anti-rheumatic activity and ability to increase glutathione concentration in the liver of male rats. This contention is reinforced by the unquestionable therapeutic superiority of salicylate, since this compound was outstanding in its effect, in single dose, on liver glutathione.

The significance of the apparent relation between anti-rheumatic activity and effect on rat liver glutathione is obscure. The metabolic reactivity and rapid turnover rate of rat liver glutathione (Waelsch & Rittenberg, 1941) suggest that changes in its concentration simply reflect variation in the volume and quality of metabolic activity for which glutathione is necessary. The present findings therefore imply the existence of a common metabolic effect after the administration of salicylate, gentisate or benzoate, and it is suggested that this primary metabolic

effect, although hypothetical and undefined, may be relevant to the anti-rheumatic action of these compounds.

The association between anti-rheumatic activity and effect on rat liver glutathione is not confined to the benzoate series, but extends to antipyrine. Antipyrine is pharmacologically an inert compound (Greenberg, 1950), which, like benzoate and gentisate, lacks the dramatic side actions of salicylate, yet is as effective as salicylate in rheumatic fever (Bouchut & Levrat, 1937). The effect of antipyrine on rat liver glutathione concentration differs, however, from that of the benzoate series of anti-rheumatic compounds, in the reversal of the sex difference. The underlying metabolic change induced by antipyrine is apparently not the same as that due to salicylate, benzoate or gentisate. Further investigation is necessary to clarify this matter. Nevertheless, the relevant actions of antipyrine, salicylate, benzoate and gentisate must all occur in the same metabolic field.

That two compounds as chemically diverse yet as therapeutically equivalent as antipyrine and salicylate should both have an effect on liver glutathione concentration is surely more than a coincidence. The question arises, therefore, of whether the inference drawn from the present results may be extended: does increased glutathione concentration in rat liver occur after the administration of other anti-rheumatic compounds, such as cinchophen ? May effect on glutathione concentration of rat liver be used as a screening test for therapeutic activity ?

REFERENCES

ANDREWS, M. M. (1958). The relative potencies of some substituted salicylic acids as metabolic stimulants in the intact rat. *Brit. J. Pharmacol.*, **13**, 419–423.

ARNSTEIN, H. R. V. & NEUBERGER, A. (1951). Hippuric acid synthesis in the rat. *Biochem. J.*, **50**, 154–162.

AUSTEN, F. K., RUBINI, M. E., MERONEY, W. H. & WOLFF, J. (1958). Salicylates and thyroid function, I. Depression of thyroid function. *J. clin. Invest.*, **37**, 1131–1143.

BARTLETT, M. S. (1937). Properties of sufficiency and statistical tests. *Proc. roy. Soc. A.*, **160**, 268–282.

BHATTACHARYA, S. K., ROBSON, J. S. & STEWART, C. P. (1955). The determination of glutathione in blood and tissues. *Biochem. J.*, **60**, 696–702.

BOUCHUT, L. & LEVRAT, M. (1937). L'antipyrine dans le traitement du rheumatisme articulaire aigu. *Presse méd.*, **45**, 1467–1468.

GOLDBERG, R. C., WOLFF, J. & GREEP, R. O. (1955). The mechanism of depression of plasma protein-bound iodine by 2,4 dinitrophenol. *Endocrinology*, **56**, 560–566.

GREENBERG, L. A. (1950). *Antipyrine*. New Haven: Hillhouse Press.

HENRIQUES, S. B., HENRIQUES, O. B. & MANDELBAUM, F. R. (1957). Incorporation of glycine into glutathione and fibrinogen of rats under adrenaline treatment. *Biochem. J.*, **66**, 222–227.

HORDER, Lord & BYWATERS, E. G. L. (1955). In *Textbook of the Rheumatic Diseases*, p. 148, ed. COPEMAN, W. S. C. Edinburgh: Livingstone.

LAZOROW, A. (1954). In *Glutathione : A Symposium*, p. 231, ed. COLOWICK, S. P. and others. New York: Academic Press.

LUTWAK-MANN, C. (1942). The effect of salicylate and cinchophen on enzymes and metabolic processes. *Biochem. J.*, **36**, 706–728.

MEADE, B. W. (1954). Effect of certain hydroxybenzoic acids on oxygen consumption of Wistar rats. *Ann. rheum. Dis.*, **13**, 60–62.

PATTERSON, J. W. & LAZOROW, A. (1955). In *Methods of Biochemical Analysis*, p. 259, ed. GLICK, D. New York: Interscience.

POTTER, V. R. & ELVEJHEM, C. A. (1936). A modified method for the study of tissue oxidations. *J. biol. Chem.*, **114**, 495–504.

QUILLEY, E. & SMITH, M. J. H. (1952). The application of paper partition chromatography to the study of the metabolism of salicylate in the rat. *J. Pharm.* (*Lond.*), **4**, 624–630.

SCHROEDER, E. F. & WOODWARD, G. E. (1939). A titrimetric modification of the glyoxalase method for the estimation of reduced glutathione. *J. biol. Chem.*, **129**, 283–294.

SNEDECOR, G. W. (1956). *Statistical Methods*, 5th ed. Ames: Iowa State College Press.

STOCKMAN, R. (1920). *Rheumatism and Arthritis*, p. 39. Edinburgh: Green.

WAELSCH, H. & RITTENBERG, D. (1941). Glutathione, I. The metabolism of glutathione studied with isotopic glycine. *J. biol. Chem.*, **139**, 761–774.

WOLFF, J. & AUSTEN, F. K. (1958). Salicylates and thyroid function, II. The effect on the thyroid-pituitary interrelation. *J. clin. Invest.*, **37**, 1144–1152.

WOLFF, J., RUBIN, L. & CHAIKOFF, I. L. (1950). The influence of 2,4-dinitrophenol on plasma protein-bound iodine. *J. Pharmacol. exp. Ther.*, **98**, 45–48.

WOODWARD, G. E. (1935). Glycxalase, III. Glyoxalase as a reagent for the quantitative micro-estimation of glutathione. *J. biol. Chem.*, **109**, 1–10.

Brit. J. Pharmacol. (1961), **16**, 188–194.

RESPONSES OF THE ISOLATED SYMPATHETIC NERVE-DUCTUS DEFERENS PREPARATION OF THE GUINEA-PIG

BY

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An isolated ductus deferens preparation together with the sympathetic hypogastric nerve of the guinea-pig is described. While the preparation showed no spontaneous contractions, nerve stimulation produced longitudinal contractions. The responses to successive periods of nerve stimulation remained constant for several hours. After noradrenaline had been added to the bath fluid for 30 min and then the fluid repeatedly changed to wash out the noradrenaline, the effect of nerve stimulation was greatly increased but declined in the course of 1 hr to the original height. Cocaine also increased the response to stimulation. Preparations made from guinea-pigs which had previously received reserpine responded to nerve stimulation with progressively diminishing contractions. In these circumstances, the administration of noradrenaline restored the response to nerve stimulation to that seen originally.

This paper describes the effect of noradrenaline and cocaine on the response to electrical stimulation of the nerve to the isolated ductus deferens preparation of the guinea-pig.

METHODS

Guinea-pigs were killed by a blow on the head and bled out. The animal was fixed on its back on the operating table, the abdomen opened in the mid-line and the gut displaced to the right. The testes were pushed into the abdominal cavity by pressure on the scrotum. Holding one testis, the ductus deferens was freed from connective tissue and cut from the epididymis. The testis was then removed. Grasping the cut end of the ductus deferens with small forceps, it was separated from the adjacent tissue. Without further cleaning of the ductus deferens, the hypogastric nerves were identified. The right and left nerves can easily be seen in the middle of the mesentery of the colon. One nerve was tied, and cut 5 cm from the ductus deferens; this was cleaned to within 0.5 cm of the organ. The remainder of the nerve up to the ductus deferens, which is fine and diffuse, was preserved by isolating the piece of peritoneum which contained it. The ductus deferens was then cut from the urethra and removed together with its nerve and the small piece of peritoneum to a bath of Krebs solution. During the dissection the organ and nerve were moistened with Krebs solution. A second satisfactory preparation can be made from the contralateral side, for speed in preparation is not essential.

The ductus deferens was tied by its proximal end to a hollow glass rod and set up in an isolated organ bath containing 12 ml. of Krebs solution at 31° C bubbled with 5% carbon dioxide and 95% oxygen. The distal end of the ductus was attached to a lever which wrote on a smoked paper. The hypogastric nerve was passed through the channel of a pair of electrodes of the pattern described by Burn & Rand (1960a) situated below the meniscus of the bath fluid. A vibrator attached to the frame of the organ bath improved recordings. The preparation was stimulated submaximally by rectangular pulses of 2 to 3 V and of 2 msec

duration at a frequency of 80/sec. The preparation was stimulated for 2 to 3 sec every min. Similar preparations can be made from rabbits, rats and mice.

The composition of the Krebs solution used was: NaCl 6.60 g, KCl 0.35 g, $CaCl_2$ 0.28 g, KH_2PO_4 0.162 g, $MgSO_47H_2O$ 0.294 g, dextrose 2.08 g, $NaHCO_3$ 2.10 g, in 1 l. of distilled water.

RESULTS

When set up in Krebs solution, the preparation from the guinea-pig exhibited no spontaneous contractions. Contractions of the ductus deferens with repetitive electrical stimulation of the hypogastric nerve were quick and strong, and are

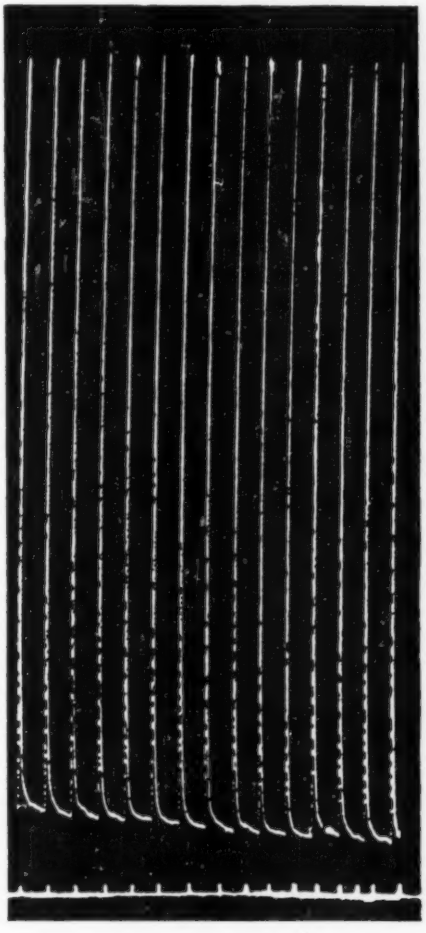

FIG. 1

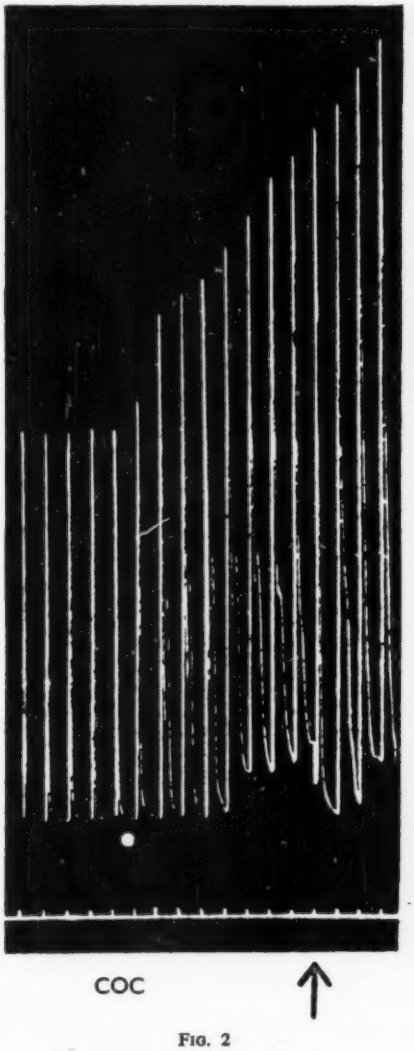

FIG. 2

Fig. 1. Record of the contractions of the isolated ductus deferens preparation of the guinea-pig in response to submaximal stimulation (2 V, 2 msec, 80 pulses/sec) of the hypogastric nerve for 2 sec in each min. Time, min.

Fig. 2. Records as in Fig. 1. At the white dot (COC) cocaine was added to the bath in a concentration of 15×10^{-6} g/ml. At the arrow the bath was washed out. Time, min.

shown in Fig. 1. There was very little decrease in the height of the responses when contractions were produced at intervals of 1 min for several hours. The responses of preparations isolated from rabbits, rats and mice were similar.

Cocaine. The effect of cocaine (15×10^{-6} g/ml.) on the response to nerve stimulation was observed on 12 preparations each from different guinea-pigs. In 5 experiments the response to stimulation was greatly increased. Fig. 2 shows that the effect of cocaine developed gradually and continued after washing, at the arrow.

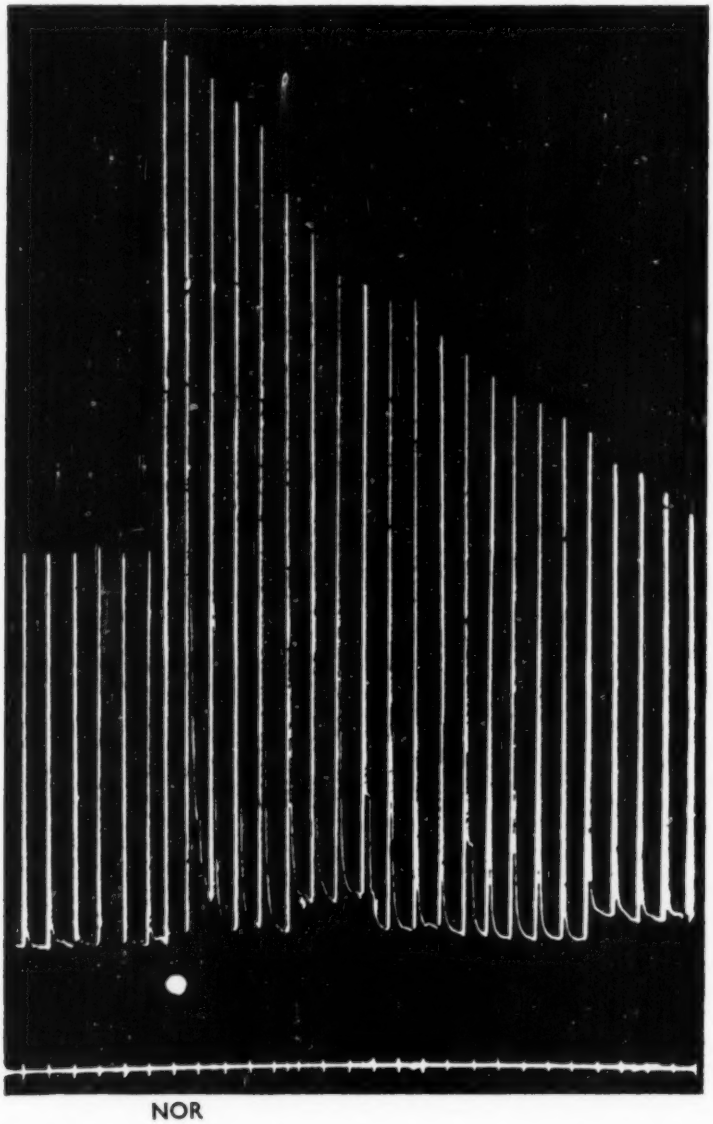

Fig. 3. Record as in Fig. 1. At the white dot (NOR) the kymograph was stopped, stimulation suspended and noradrenaline (15 μg/ml.) was added. After 30 min, the bath was washed out 5 times at 3-min intervals, the kymograph was then re-started and stimulation resumed. Time, min.

In 4 experiments the increase was small and in 3 there was a decrease. However, in these 7 experiments, the contractions after washing out the cocaine were greater than before its administration.

Noradrenaline. Noradrenaline (15 μg/ml.) left in contact with the guinea-pig ductus deferens preparation for 30 min caused a contraction. On washing every 3 min for 15 min, the ductus relaxed nearly to the original base line. The height of the response to nerve stimulation after treatment with noradrenaline was always found to be at least twice as great as that seen before the application of noradrenaline (Fig. 3).

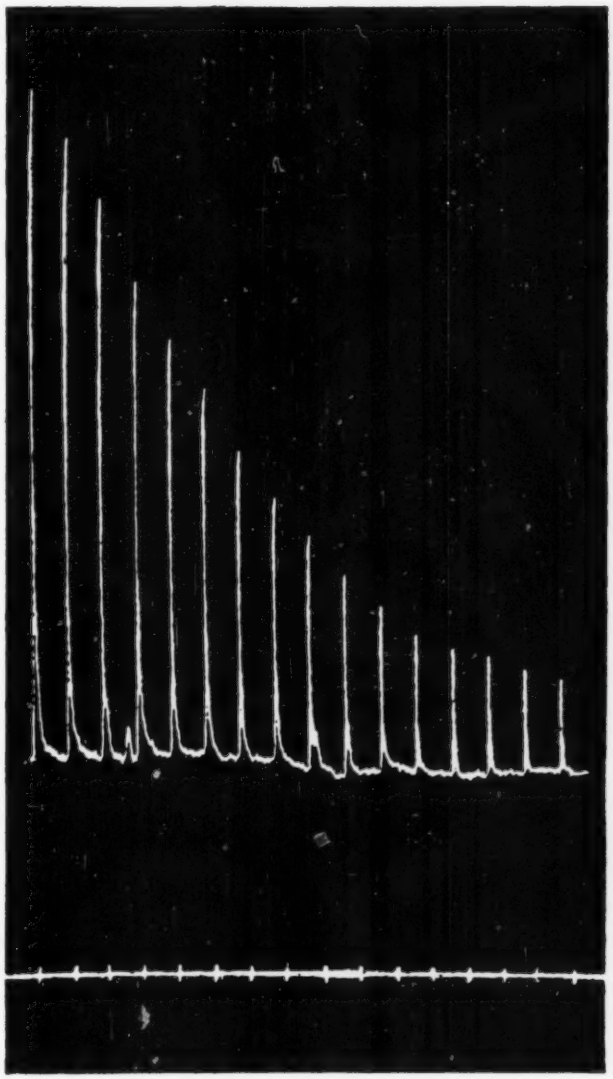

Fig. 4. Record as in Fig. 1. The ductus deferens preparation was made from a guinea-pig treated for 2 days previously with reserpine (see text). Note the progressive decrease in response to stimulation. Time, min.

Reserpine. Preparations of the ductus deferens were made from guinea-pigs which had received intraperitoneally for the 2 days previously 0.5 mg/kg of reserpine on the first day and 1.0 mg/kg on the second day. In all these preparations a progressive decrease in the response to stimulation was seen after the first 2 or 3 periods of stimulation, but the contractions were never completely abolished (Fig. 4).

Effect of noradrenaline after reserpine. Fig. 5a shows small and regular contractions in response to nerve stimulation of a ductus from a reserpine-treated guinea-pig. Noradrenaline (15 μg/ml.) added to the bath caused a marked contraction. The noradrenaline was left in the bath for 30 min. After washing every 3 min for 15 min, the ductus relaxed nearly to the base line. The response to nerve stimulation was then seen to be increased to more than 4 times its initial height (Fig. 5b). The

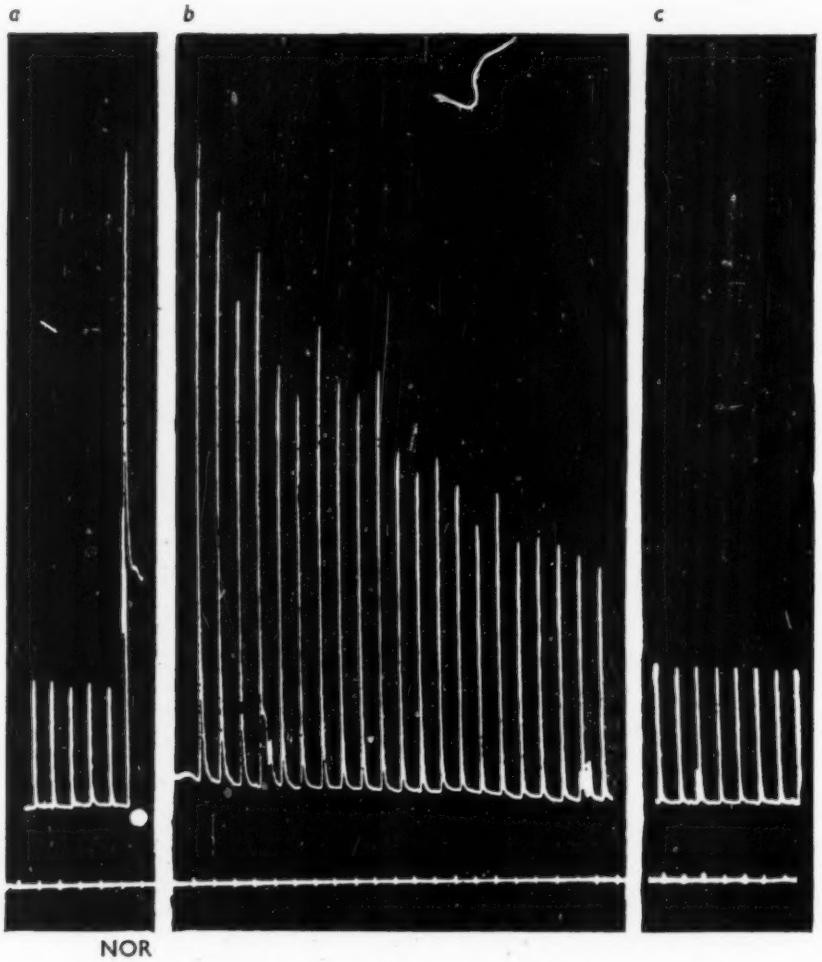

Fig. 5. Records as in Fig. 1 from a ductus deferens preparation of a guinea-pig previously treated with reserpine (see text). (*a*) At the white dot (NOR) noradrenaline (15 μg/ml.) was added and then washed out as in Fig. 3. (*b*) When stimulation was resumed the contractions were at first enhanced, but in (*c*) 60 min later they had declined to their initial heights.

potentiated responses to stimulation gradually decreased, until 1 hr later they were no larger than those before noradrenaline (Fig. 5c).

The potentiation of the responses to stimulation of the nerve by noradrenaline both in the normal and in the reserpine-treated preparation was much greater than that seen after resting the preparation for 45 min.

DISCUSSION

Stimulation of the hypogastric nerve of rabbits causes contractions of the uterine cornu and cervix *in situ* (Schofield, 1952) and of the cornu *in vitro* (Varagic, 1956). Mann & West (1951) recovered adrenaline and noradrenaline from blood in the ovarian vein after stimulation of hypogastric nerves to the uterus of the cat. In most species the sympathetic outflow to the uterus is motor and the chief route is through the hypogastric nerves (Garry, 1957). These same sympathetic hypogastric nerves in the male supply motor innervation to the ductus deferens. The preparation of the isolated ductus deferens with its hypogastric nerve described here is easy to make, durable, and gives uniform contractions to nerve stimulation.

Schofield (1952) found that cocaine did not increase the contraction of the rabbit uterus in response to stimulation of the hypogastric nerve *in situ*. However, in the *in vitro* preparation of the rabbit uterus, Varagic (1956) found an increased response. In nearly half of the present experiments with the guinea-pig ductus deferens-hypogastric nerve preparation, cocaine potentiated responses ; but in some experiments it caused inhibition. However, after washing out cocaine from the bath the potentiating effect due to cocaine was then seen. It is possible that inhibition, when it occurred, was due to the local anaesthetic effect of cocaine on the hypogastric nerve.

Stores of noradrenaline have been detected in a number of tissues, such as the heart (Bertler, Carlsson & Rosengren, 1956), arteries (Burn & Rand, 1958), iris and spleen (Burn & Rand, 1959) and skin of the cat's tail (Burn, Leach, Rand & Thompson, 1959). These stores of noradrenaline can be depleted by treatment with reserpine and then the usual responses to sympathetic stimulation are reduced or abolished (Muscholl & Vogt, 1958 ; Burn, Leach, Rand & Thompson, 1959 ; Hukovic, 1959). The results with ductus preparations from reserpine-treated guinea-pigs are compatible with a similar depletion of the noradrenaline stores of the ductus, so that stimulation of the nerve could only release small amounts of noradrenaline from the reduced store.

After treating the ductus preparation with noradrenaline, the responses to hypogastric nerve stimulation were enhanced. This observation is in accord with the findings of others that noradrenaline enhanced or restored the responses to sympathetic nerve stimulation (Gillespie & Mackenna, 1959 ; Burn & Rand, 1960a and b). The effect of noradrenaline potentiating responses to sympathetic stimulation may be due to a reloading of the depleted stores ; this suggestion is supported by the observation of Pennefather & Rand (1960) that an infusion of noradrenaline increased the noradrenaline content of the uterus and kidney of the cat.

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REFERENCES

BERTLER, Å., CARLSSON, A. & ROSENGREN, E. (1956). Release by reserpine of catechol amines from rabbits' hearts. *Naturwissenschaften*, **43**, 521.

BURN, J. H. & RAND, M. J. (1958). Noradrenaline in artery walls and its dispersal by reserpine. *Brit. med. J.*, **1**, 903–908.

BURN, J. H. & RAND, M. J. (1959). The cause of the supersensitivity of smooth muscle to noradrenaline after sympathetic degeneration. *J. Physiol.* (*Lond.*), **147**, 135–143.

BURN, J. H. & RAND, M. J. (1960a). The relation of circulating noradrenaline to the effect of sympathetic stimulation. *J. Physiol.* (*Lond.*), **150**, 295–305.

BURN, J. H. & RAND, M. J. (1960b). The effect of precursors of noradrenaline on the response to tyramine and sympathetic stimulation. *Brit. J. Pharmacol.*, **15**, 47–55.

BURN, J. H., LEACH, E. H., RAND, M. J. & THOMPSON, J. W. (1959). Peripheral effects of nicotine and acetylcholine resembling those of sympathetic stimulation. *J. Physiol.* (*Lond.*), **148**, 332–352.

GARRY, R. C. (1957). Innervation of abdominal viscera. *Brit. med. Bull.*, **13**, 202–206.

GILLESPIE, J. S. & MACKENNA, B. R. (1959). The effect of reserpine on the response of the rabbit ileum and colon to stimulation of their extrinsic nerves *in vitro*. *J. Physiol.* (*Lond.*), **147**, 31–32P.

HUKOVIĆ, S. (1959). Isolated rabbit atria with sympathetic nerve supply. *Brit. J. Pharmacol.*, **14**, 372–376.

MANN, M. & WEST, G. B. (1951). The nature of uterine and intestinal sympathin. *Brit. J. Pharmacol.*, **6**, 79–82.

MUSCHOLL, E. & VOGT, M. (1958). The action of reserpine on the peripheral sympathetic system. *J. Physiol.* (*Lond.*), **141**, 132–155.

PENNEFATHER, J. N. & RAND, M. J. (1960). The increase in the content of noradrenaline in tissues after the infusion of noradrenaline, dopamine and L-DOPA. *J. Physiol.* (*Lond.*), **154**, 277–287.

SCHOFIELD, B. M. (1952). The innervation of the cervix and cornu uteri in the rabbit. *J. Physiol.* (*Lond.*), **117**, 317–328.

VARAGIĆ, V. (1956). An isolated rabbit hypogastric-nerve-uterus preparation, with observations on the hypogastric transmitter. *J. Physiol.* (*Lond.*), **132**, 92–99.

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EFFECT OF DOPAMINE ON THE ISOLATED PERFUSED LUNG LOBES OF THE DOG

BY

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Dopamine (β-3:4-dihydroxyphenylethylamine) has a pressor action on the pulmonary vascular bed in isolated perfused lung lobes of the dog. Its pulmonary vasoconstrictor effect in this preparation is about 1/20–1/30 that of adrenaline or noradrenaline. When compared with adrenaline, dopamine is less effective as a vasoconstrictor in the bronchial vascular system than in the pulmonary circulation. Even very large doses of dopamine had no bronchomotor effect in the isolated perfused lung lobes. Addition of dopamine alone, or of dopamine together with some " new " blood, sometimes caused a potentiation of the pulmonary vasoconstrictor responses to sympathetic nerve stimulation.

The demonstration of a relatively large dopamine (β-3:4-dihydroxyphenylethylamine) content in lung tissue by Euler & Lishajko (1957) and by Schümann (1958) has some interesting aspects. According to these investigators, more than 90% of the total amount of catecholamines in lung tissue is dopamine, and it is possible that it may have functions other than serving as precursor in local noradrenaline formation. Schümann (1959) has speculated that dopamine may have its own functions and act as a local hormone. It was therefore thought of interest to test the effect of dopamine when introduced into the circulation of isolated perfused lungs. The preparation used was one in which both the pulmonary and the bronchial circulations were perfused (Daly & Waaler, 1960; Allison, Daly & Waaler, 1961). Since in the dog the bronchial vascular system supplies several important intrapulmonary structures such as the nerves and the airways down to the respiratory bronchioles, an adequate perfusion of this system may be of great importance for the reactivity of the preparation to drugs. It is well known that vasomotor responses to sympathetic nerve stimulation cannot be obtained in such a preparation unless the bronchial vascular system is being perfused (Daly & Euler, 1932; Allison, Daly & Waaler, 1961). It should also be emphasized that a preparation of this type can be perfused for several hr at a pulmonary vascular resistance which is well within the range for the normal resting dog (Daly & Waaler, 1960). This allows testing of vasomotor drugs to be carried out during periods in which the vascular tone in the pulmonary circulation is probably not much different from that occurring *in vivo*.

METHODS

Mongrel dogs (11 to 29 kg) were premedicated with morphine sulphate (2 mg/kg) and injected intravenously with heparin (Boots' powdered heparin, 3 mg/kg) and in most experiments also

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with atropine sulphate (1 mg). They were then exsanguinated through a cannula which under local anaesthesia had been inserted into a femoral artery. The blood was collected in a glass cylinder containing heparin 6 mg/kg body weight, the total amount of heparin used thus being about 9 mg/kg body weight. Penicillin ("Crystapen" crystalline penicillin G, Glaxo, 5 i.u./ml. blood) was added, the blood was filtered through a piece of linen and stored at 36° C in stoppered bottles until needed for perfusion.

The left apical and the left cardiac lobes, together with the heart and its attached vessels, including the descending aorta with the segregated bronchial artery or arteries (which in the dog come off from the 5th to 8th intercostal arteries on the right side), were removed from the body and arranged for perfusion in a warmed perspex tray. The left thoracic vagosympathetic nerve was also preserved and included in the preparation.

Perfusion. The pulmonary circulation was perfused either at a constant head of pressure or at a constant volume inflow through a cannula tied into the pulmonary artery. The outflowing blood was collected through a cannula inserted into the left auricle and the flow measured by leading this blood through a modified Gaddum flow recorder. The pulmonary arterial pressure was measured by a saline manometer and a Marey tambour. The bronchial circulation was perfused at a constant volume inflow through a cannula inserted into the abdominal end of the thoracic part of the aorta. All branches from the aorta, other than the bronchial artery or arteries and some small arterial twigs to the left thoracic vagosympathetic nerve, were ligated. There was one exception to this procedure: the blood flow in the reduced systemic circulation was small, and in order to prevent stagnation of the blood in the aorta an arterial branch was cannulated and a controlled amount of blood from it allowed to spill back into the reservoir. The perfusion pressure in the systemic circuit was measured by a mercury manometer. Perfusion of the pulmonary circulation and of the reduced systemic circulation were carried out by two Dale-Schuster pumps, which drew blood from a common blood reservoir with a volume of about 250 ml. The total volume of the external perfusion circuits was about 450 ml.

Ventilation. Positive pressure ventilation with 6% carbon dioxide in air or 6% carbon dioxide in oxygen was carried out with a Starling "Ideal" respiration pump at peak inspiratory pressures of 80 to 95 mm of water and an expiratory pressure of 30 mm of water. The tidal air was controlled by measuring the ventilation overflow according to the method of Konzett & Rössler (1940).

Dopamine hydrochloride (California Corporation for Biochem. Research), (−)-noradrenaline bitartrate ("Levophed," Bayer, London) and adrenaline chloride (Parke, Davis, Hounslow) were used. All doses are referred to in terms of the bases. The substances were dissolved in small volumes of 0.9% sodium chloride solution and either added to the blood reservoir or injected into the pulmonary arterial inflow tubing. The amount of saline in which injected doses of the substances were dissolved was kept constant in each experiment, usually being 0.1 to 0.3 ml.

Pulmonary vascular resistance. The pulmonary vascular resistance, corresponding to the whole of both lungs, was calculated as previously described (Daly & Waaler, 1960).

RESULTS

Circulatory effects. When added to the blood reservoir or injected into the pulmonary arterial inflow tubing, dopamine caused pulmonary vasoconstriction. Its vasoconstrictor effect was less marked, however, than that of noradrenaline or adrenaline. The ratio of equipotent doses of noradrenaline, adrenaline and dopamine was about 1:1:25. Various preparations differed in the sensitivity of their pulmonary vascular bed to all the three amines, but the relative sensitivity to each of them remained the same. The responses in one preparation to small doses of adrenaline, noradrenaline and dopamine injected into the pulmonary arterial inflow tubing are shown in Fig. 1. Fig. 2 shows the responses in another preparation to

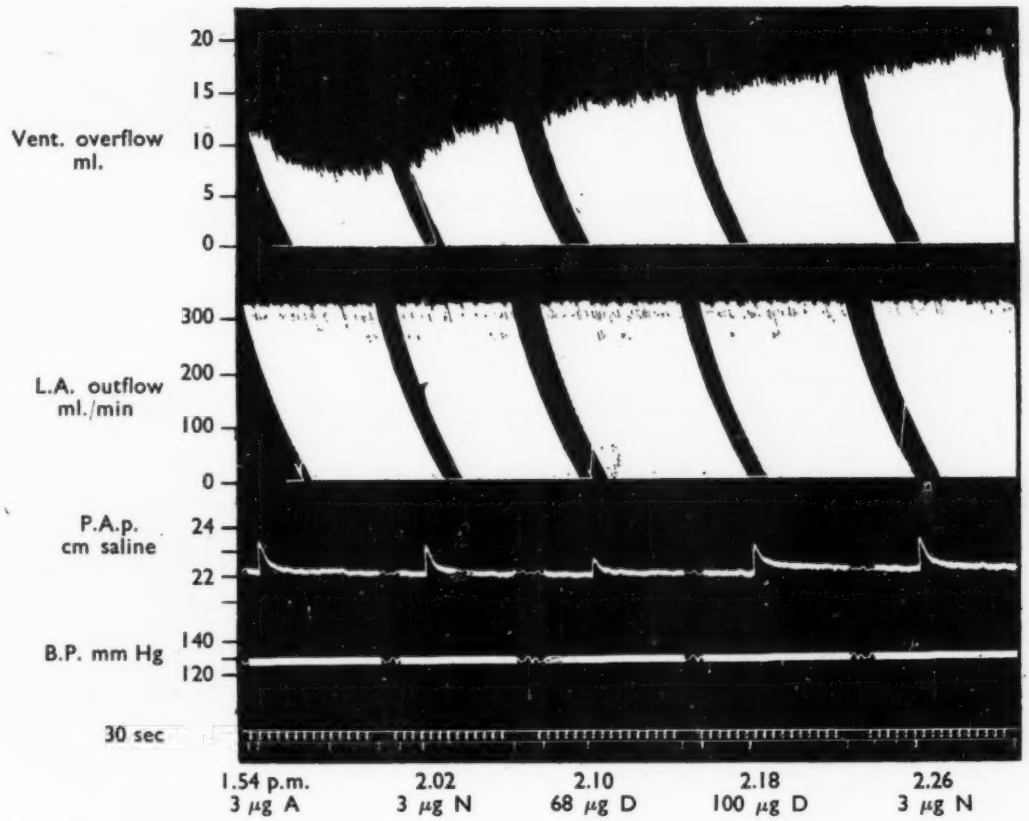

Fig. 1. Effect of injections of adrenaline (A), noradrenaline (N) and dopamine (D) into pulmonary artery of isolated dog lung perfused at constant volume inflow. Dog: ♂, 23 kg, left apical and left cardiac lobes perfused. Tracings from above: ventilation (Vent.) overflow (respiration pump stroke=70 ml.), left atrial (L.A.) blood outflow, pulmonary arterial perfusion pressure (P.A.p.), perfusion pressure in reduced systemic circulation (i.e. aorta and bronchial circulation) (B.P.) and time marker and signals. Left atrial pressure=3.5 cm of saline. All doses of catecholamine were dissolved in 0.15 ml. of 0.9% sodium chloride solution and injected in the course of 5 sec.

additions of the three amines to the blood reservoir. Vasoconstrictor responses of similar duration and magnitude were obtained in preparations perfused at constant pressure.

The additions to the perfusate in the reservoir of dopamine caused rather prolonged vasoconstrictor responses in this isolated lung preparation, similar to responses produced by additions of adrenaline or noradrenaline. The rate at which the responses declined is seen in Fig. 2. A dose of 0.5 to 1 mg of dopamine gave a response which needed more than 0.5 hr to disappear.

It will be seen from Fig. 2 that doses of dopamine which caused a definite constriction in the pulmonary vascular bed did not have any appreciable vasoconstrictor effect in the reduced systemic (bronchial) circulation. Since the two circulations were perfused with blood from a common reservoir, to which the test substances were added, the concentration of the amines in the blood entering the two circuits was

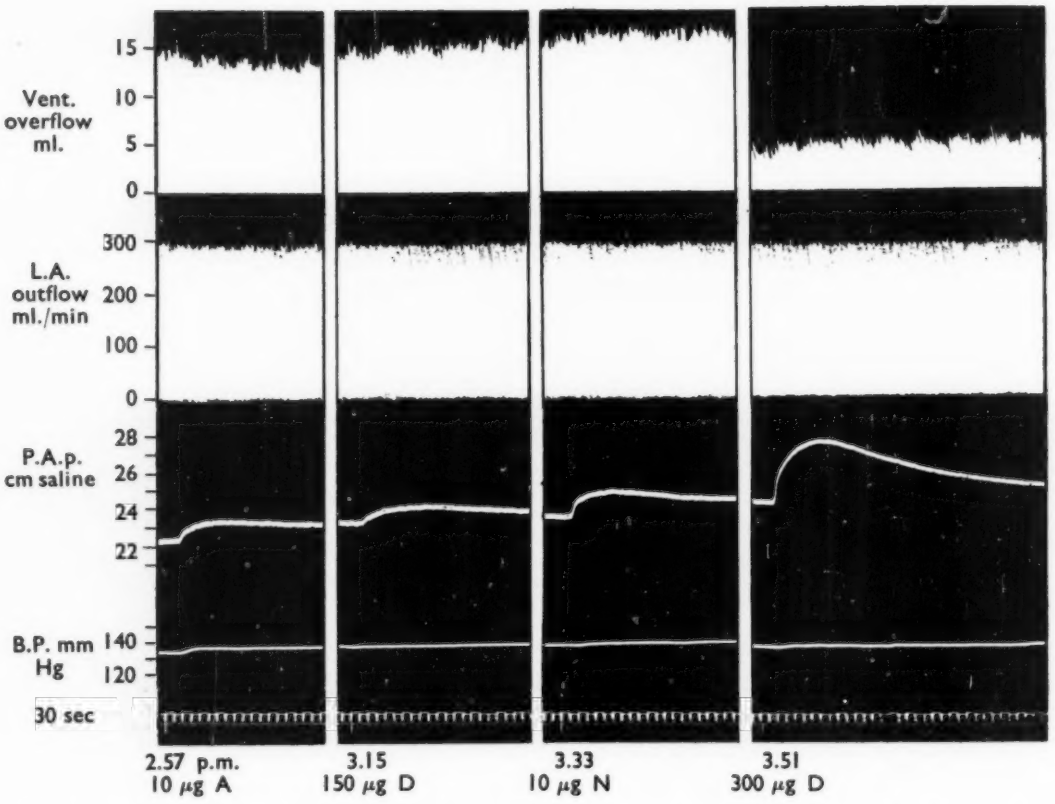

Fig. 2. Effect of adding adrenaline (A), dopamine (D) and noradrenaline (N) to the blood reservoir of an isolated dog lung perfused at constant volume inflow. Dog: ♂, 19 kg, left apical and left cardiac lobes perfused. Tracings as in Fig. 1. Respiration pump stroke = 66.5 ml. up to 3.48 p.m., then reduced to 52 ml. Left atrial pressure = 4 cm saline. All doses of catecholamine dissolved in 0.2 to 0.3 ml. of 0.9% sodium chloride solution and added to blood reservoir (volume 250 ml.) during stirring.

the same. The preparation was not suitable for quantitative evaluations of vasomotor responses in the bronchial circulation, because of the shunting of blood back from the aorta to the reservoir. This constant leak of arterial blood reduced the amplitude of recorded pressure changes caused by vasomotor events in the bronchial vascular system. In spite of this disadvantage, however, some comparison between the effect of various substances on the bronchial vascular bed was possible. It is seen from Fig. 2 that 10 μg of adrenaline had about the same vasoconstrictor effect in the pulmonary circulation as had 150 μg of dopamine. This dose of adrenaline, however, had an appreciable constrictor effect in the bronchial circulation whereas 300 μg of dopamine had hardly any effect on the perfusion pressure in this circulation. This difference between adrenaline and dopamine was observed in several preparations. Noradrenaline seemed to be intermediate to adrenaline and dopamine in its relative vasoconstrictor effect on the bronchial vascular bed.

Effect on tidal air. Dopamine never caused any changes in the tidal air of these preparations, not even when added in doses large enough to increase the pulmonary

vascular resistance by 100%. Adrenaline always gave bronchodilatation, even after many hr of perfusion (Figs. 1 and 2). Daly, Hebb & Petrovskaia (1941) reported that in a perfused lung preparation adrenaline caused bronchodilatation in the initial part of an experiment, and bronchoconstriction after approximately 2 hr of perfusion. The reason for the constant finding of a bronchodilator response to adrenaline in the experiments here described may be in some way connected with the perfusion of the bronchial vascular system. In the preparation used by Daly, Hebb & Petrovskaia (1941) the bronchial circulation was not perfused. Noradrenaline in doses large enough to cause a marked increase in pulmonary vascular resistance had no effect on the tidal air in the present preparation.

Effect on vasoconstrictor responses to sympathetic nerve stimulation. In some of the preparations, stimulation of the postganglionic sympathetic nerve fibres to the lung was carried out at 10 min intervals. The stimuli were applied to the left thoracic vagosympathetic nerve. Such stimulation regularly gives pulmonary vasoconstrictor responses in this type of preparation (Daly, 1958 ; Allison, Daly & Waaler, 1961). Because the preparation was atropinized, no bronchoconstrictor response to stimulation of the parasympathetic fibres in the vagosympathetic nerve occurred. The pulmonary vasoconstrictor effect of nerve stimulation could be shown to persist during short periods of bronchial circulation interruption. It was therefore due to

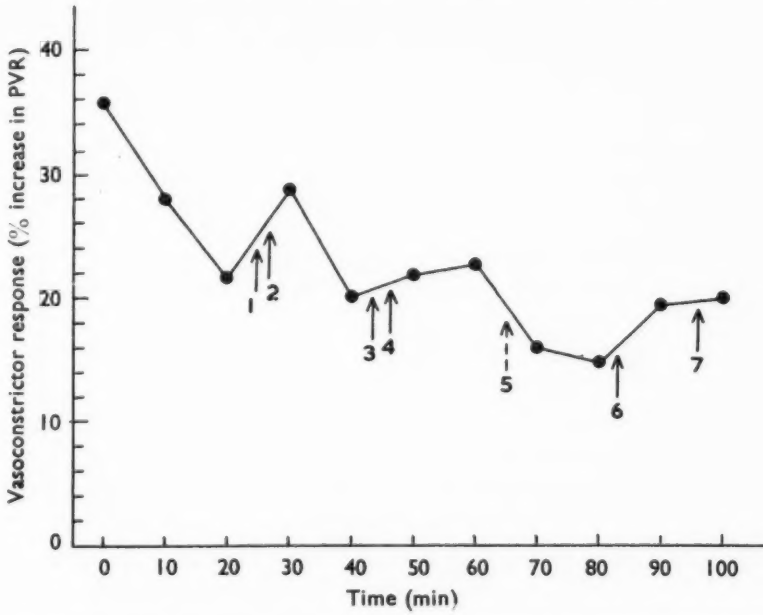

Fig. 3. Effect on declining pulmonary vasoconstrictor responses to sympathetic nerve stimulation of additions of dopamine. Isolated dog lung perfused at constant pressure. Each point represents a vasoconstrictor response to a stimulus applied to the left thoracic vagosympathetic nerve. Responses expressed as % increase in calculated pulmonary vascular resistance (PVR). Dopamine, dissolved in small amounts of 0.9% sodium chloride solution (0.1 to 0.3 ml.), added to blood reservoir during stirring. At 1 and 2, 50 μg of dopamine; at 3 and 4, 150 μg of dopamine; at 5, 110 ml. of "new" blood; and at 6 and 7 100 μg of dopamine was added. "New" blood was without effect in this preparation.

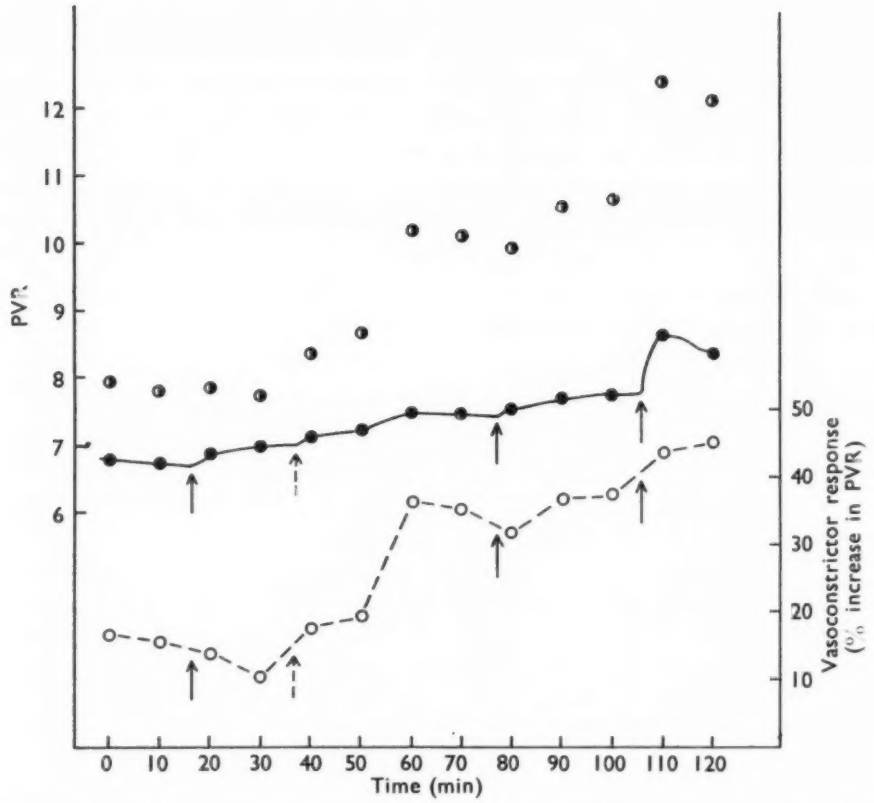

Fig. 4. Effect on declining pulmonary vasoconstrictor responses to sympathetic nerve stimulation of additions of dopamine and " new " blood. Isolated dog lung perfused at constant volume inflow. Stimuli applied to left thoracic vagosympathetic nerve. ● represents the calculated pulmonary vascular resistance (PVR) at beginning of each stimulation, ◑ the pulmonary vascular resistance at peak of vasoconstrictor response to each stimulation. In the lower curve ○ shows the vasoconstrictor responses expressed as % increase in pulmonary vascular resistance. Solid arrows=dopamine, 50 μg, 50 μg and 100 μg (in 0.1 to 0.2 ml. 0.9% sodium chloride solution), added to blood reservoir (volume 250 ml.) during stirring. Broken arrow=addition to blood reservoir of 60 ml. of " new " blood.

constriction of the pulmonary vascular bed proper, and not due to passive effects on the pulmonary circulation caused by events in the bronchial circulation (Daly, 1958). When such stimulations are carried out at 10 min intervals for some time, the marked vasoconstrictor responses which are usually obtained sometimes start to decline (Fig. 3). It was then observed that addition to the blood perfusate of dopamine 50 to 300 μg sometimes caused a moderate potentiation of the vasoconstrictor response to one, two or three of the subsequent sympathetic nerve stimulations (Fig. 3). Sometimes the very first vasoconstrictor response after dopamine addition was reduced, the following one or two showing some potentiation. Potentiation of the vasoconstrictor responses to nerve stimulation occurred after 10 out of 17 additions of dopamine in 8 experiments. The potentiating effect of dopamine was particularly marked on some occasions where dopamine and a small amount of " new " blood were added to the preparation together or one after the other. " New "

blood denotes blood which had been stored at 36° C since death of the animal. This " new " blood sometimes caused potentiation of the vasoconstrictor responses to sympathetic nerve stimulation when added alone, but even when it failed to do so a subsequent addition of dopamine would usually initiate a potentiation of the responses. On two occasions the opposite sequence of events was observed; addition of dopamine failed to potentiate the responses, but a subsequent addition of " new " blood initiated such potentiation (Fig. 4). Further addition of dopamine then caused further potentiation of the responses.

Continuous infusion of dopamine 5 to 25 μg/min caused no potentiation of the vasoconstrictor responses to sympathetic nerve stimulation. On the contrary, the responses tended to become less marked during the period of infusion. On stopping the infusion the responses regained their pre-infusion size in one, showed a small potentiation in another, and continued to decline in the third experiment.

DISCUSSION

Dopamine has a definite vasoconstrictor effect on the pulmonary vascular bed in isolated perfused dog lungs. It is considerably less effective than noradrenaline and adrenaline. When compared with adrenaline, however, dopamine seems to be a less potent vasoconstrictor in the bronchial vascular system than in the pulmonary vascular bed. This difference might have a bearing on the possible role of dopamine in the lungs.

The vasoconstrictor effect of dopamine in isolated perfused lungs wears off slowly and at about the same rate as that of noradrenaline and adrenaline. The rate of destruction of catecholamines in this isolated perfused lung preparation must be fairly slow since infusion of as little as 5 μg/min of dopamine caused a gradual rise in pulmonary vascular resistance.

The effects of dopamine on the pulmonary vasoconstrictor response to sympathetic nerve stimulation are interesting. During continuous infusion of dopamine, and sometimes also just after the addition of one single dose, the responses were diminished. This is compatible with the view that some dopamine might then be fixed to receptor sites and prevent the vasoconstrictor transmitter from acting when released by nerve stimulation (Burn & Rand, 1958). One explanation of the potentiating effect on these responses produced by dopamine, alone or in combination with " new " blood, which was observed in some of the preparations, is that dopamine was taken up at the transmitter site and transformed to noradrenaline, which may be the sympathetic transmitter in the organ. Since " new " blood and dopamine when added together sometimes caused a very marked potentiating effect on the vasoconstrictor response to sympathetic nerve stimulation, this blood might have provided an enzyme or other material necessary for such uptake and/or transformation. Speculating on these lines it would be expected that if perfused lungs became deficient in sympathetic transmitter they would give only weak vasoconstrictor responses to nerve stimulation. If, however, this deficiency were remedied by addition of dopamine and of some additional components in " new " blood, then the responses might be potentiated, as was indeed found in some of the experiments.

The reason why dopamine and "new" blood, either separately or together, sometimes failed to potentiate the vasomotor responses to nerve stimulation is obscure. It may be that the transmitter mechanism was in these experiments already working under optimal conditions.

Whatever the underlying mechanisms responsible for the potential ability of dopamine to restore the declining vasoconstrictor response to sympathetic nerve stimulation, these observations suggest a close relationship between dopamine and the transmitter apparatus concerned.

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REFERENCES

ALLISON, P. R., DALY, I. DE BURGH & WAALER, B. A. (1961). Bronchial circulation and pulmonary vasomotor nerve activity. *J. Physiol.* (*Lond.*). In the press.

BURN, J. H. & RAND, M. J. (1958). Vasomotor reversal and reserpine. *J. Physiol.* (*Lond.*), **143**, 73–74*P*.

DALY, I. DE BURGH (1958). Intrinsic mechanisms of the lung. *Quart. J. exp. Physiol.*, **43**, 2–26.

DALY, I. DE BURGH & EULER, U. S. VON (1932). The functional activity of the vasomotor nerves to the lungs in the dog. *Proc. Roy. Soc. B.*, **110**, 92–111.

DALY, I. DE BURGH, HEBB, C. O. & PETROVSKAIA, B. (1941). Adrenaline bronchoconstriction in isolated blood perfused lungs. *Quart. J. exp. Physiol.*, **31**, 129–159.

DALY, I. DE BURGH & WAALER, B. A. (1960). Bronchial circulation and pulmonary vascular resistance (PVR) in isolated perfused lung lobes. *J. Physiol.* (*Lond.*), **153**, 14*P*.

EULER, U. S. VON & LISHAJKO, F. (1957). Dopamine in mammalian lung and spleen. *Acta physiol. pharmacol. neerl.*, **6**, 295–303.

KONZETT, H. & RÖSSLER, R. (1940). Versuchsanordnung zu Untersuchungen an der Bronchialmuskulatur. *Arch. exp. Path. Pharmak.*, **195**, 71–74.

SCHÜMANN, H. J. (1958). Über den Hydroxytyramin- und Noradrenalingehalt der Lunge. *Arch. exp. Path. Pharmak.*, **234**, 282–290.

SCHÜMANN, H. J. (1959). Über den Hydroxytyramingehalt sympathischer Nerven und sympatisch innervierter Organe. *Arch. exp. Path. Pharmak.*, **236**, 44–46.

Brit. J. Pharmacol. (1961), **16**, 203–208.

METABOLISM OF [^{14}C]-HISTAMINE IN HEART-LUNG-LIVER PREPARATIONS OF CATS

BY

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The metabolism of injected [^{14}C]-histamine was studied in heart-lung-liver preparations and heart-lung preparations from cats. When the liver was included in the circulation the injected histamine was rapidly eliminated from the blood, some of its metabolites appearing in the blood within 2 min after the injection. More than 70% of the injected histamine was metabolized to 1-methylimidazol-4-ylacetic acid. In the heart-lung preparations [^{14}C]-histamine disappeared much more slowly from the blood and the major metabolite was 4-(2-aminoethyl)-1-methylimidazole (methylhistamine). Less than 4% of the injected ^{14}C was recovered in the form of imidazol-4(or 5)-ylacetic acid. In none of the experiments was there any measurable formation of acetylhistamine.

The inactivation of histamine in liver perfusion experiments has been studied by Dale & Laidlaw (1911), Guggenheim & Loeffler (1916) and Best & McHenry (1930). The latter authors found that the perfused canine liver could inactivate 10 to 60 mg histamine in 4 hr. The mechanism responsible for the disappearance of histamine from the perfusion fluid was not further investigated. It would seem that in man, dog and cat the liver contains little diamine oxidase type of histaminase (Zeller, 1942). However, as first shown by Kobayashi & Schayer (1956), the liver may have other means of inactivating histamine. These authors incubated [^{14}C]-histamine with a homogenate of cat liver and found that the histamine was methylated on the ring nitrogen remote from the side-chain. The methylhistamine formed was to a large extent oxidized to methylimidazolylacetic acid.

The properties of the histamine methylating enzyme in the liver have been elucidated in *in vitro* experiments by Lindahl (1958 and 1960) and by Brown, Tomchick & Axelrod (1959). The latter authors also demonstrated the presence of the enzyme in other tissues, for example, heart and lung.

In the present experiments the capacity of the liver, the heart and the lungs to take up and metabolize [^{14}C]-histamine injected into the blood was studied in heart-lung-liver preparations of cats.

METHODS

Female cats weighing 2.2 to 3.0 kg were anaesthetized by an intraperitoneal injection of pentobarbitone sodium (Nembutal, Abbott) in a dose of 50 mg/kg. The heart-lung-liver preparations and heart-lung preparations were made with the technique described by Örskov (1953). Histamine labelled with ^{14}C in position 2 of the imidazole ring was injected into the portal vein in the heart-lung-liver preparations and into the inferior vena cava in the heart-lung

preparations. The amount injected was 32 μg base with a specific activity of 42.4 μc/mg (purchased from the Radiochemical Centre, Amersham, England). The histamine was dissolved in 5 ml. saline containing glucose 1 mg/ml. The injection time was about 10 sec. Arterial blood was collected from a polythene catheter in the aorta. The arterial blood samples (10 ml. each) were taken 0.5, 2, 10 and 20 min after the injection of histamine. At about 25 min after the injection of histamine trypan blue was injected by the same route in order to demonstrate any major leakage of blood from the heart-lung-liver preparation to the other tissues of the experimental animal. Judged in this way, there was no major leakage. The only place outside the heart, lung and liver where the colour was visible was in some small blood vessels in the diaphragm. At 30 min after the injection of histamine the preparation was bled out. The volume of the collected blood was measured and aliquots taken for isotope dilution assays similar to those made on the preceding samples of arterial blood.

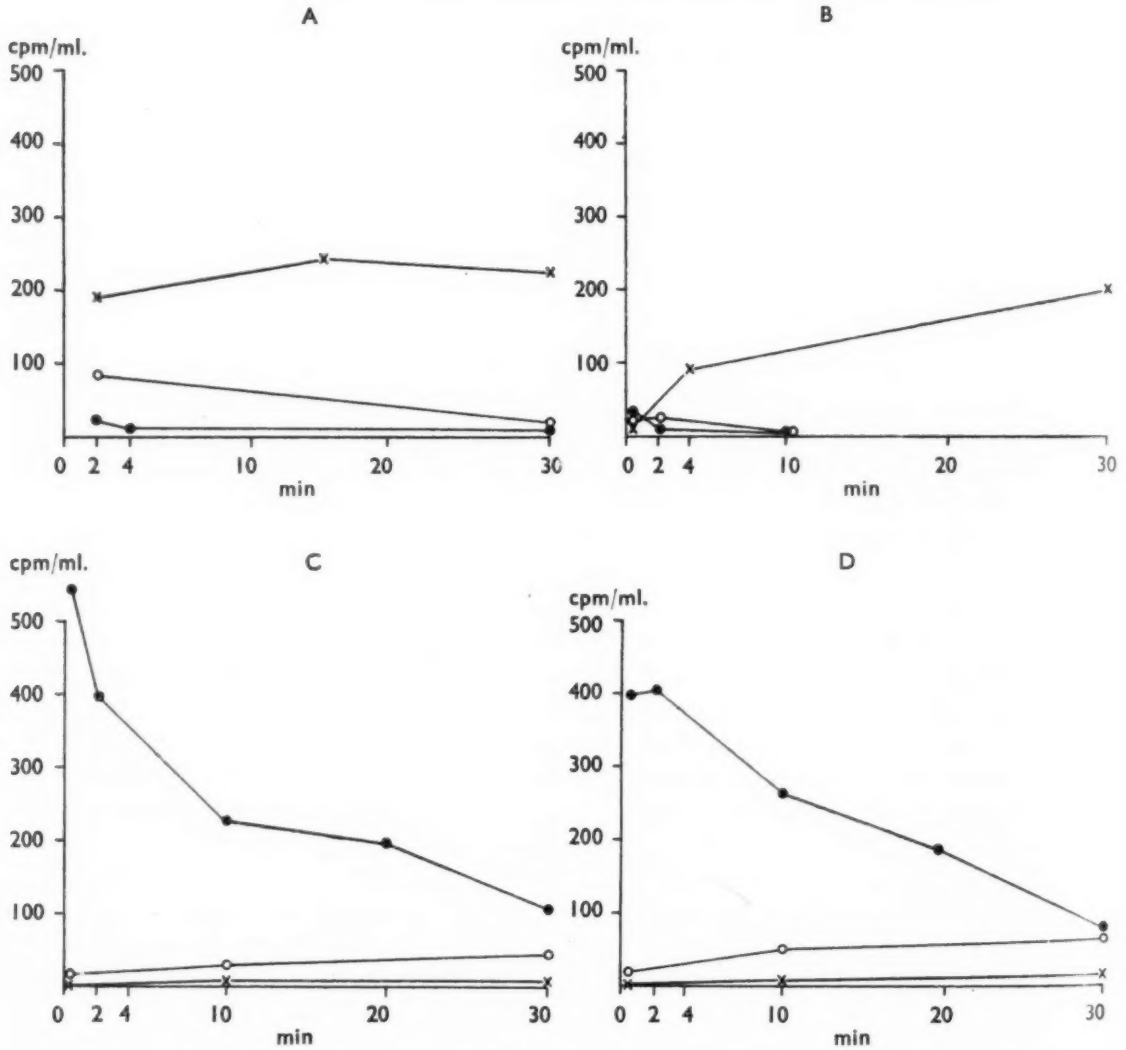

Fig. 1. The concentrations of labelled histamine (●——●), methylhistamine (○——○) and methylimidazoleacetic acid (X——X) in arterial blood at various times after the injection of 32 μg [^{14}C]-histamine into the portal vein of heart-lung-liver preparations from cats (Expts. A and B) or into the inferior caval vein in heart-lung preparations (Expts. C and D).

Isotope dilution assays for [^{14}C]*-histamine and some of its known metabolites in blood*

Each 10 ml. blood sample was divided into 2 ml. aliquots, to which the respective carrier was added immediately. The carrier was non-radioactive histamine, 4-(2-aminoethyl)-1-methylimidazole (methylhistamine), 1-methylimidazol-4-ylacetic acid (methylimidazoleacetic acid), imidazol-4(or 5)-ylacetic acid (imidazoleacetic acid), *or* acetylhistamine, all in amounts equivalent to 206 mg of the corresponding picrate. Each compound was then extracted and its radioactivity measured as described by Lindell & Schayer (1958).

Isotope dilution assays for [^{14}C]*-histamine and its metabolites in the tissues at the end of the experiment*

At the end of each experiment the heart, lungs and liver were weighed and then minced with scissors. Aliquots of 2 g were taken, to which carrier was added. The samples were then ground with sand in mortars, trichloroacetic acid being added. The extraction and measurement of radioactivity of respective compound was as mentioned above.

Metabolism of [^{14}C]*-histamine* in vitro

Lung and heart tissue from cats anaesthetized and bled out was minced with scissors and 2 g aliquots suspended in 2 ml. 0.1 M phosphate buffer (*p*H 7.4). This suspension was incubated with 0.18 μg [^{14}C]-histamine at 37° C for 2 hr. For comparison similar incubations were also carried out with feline blood instead of minced tissue. The amount of radioactive methylhistamine formed was determined as described above.

Of the labelled histamine used, 1 μg gives 2,040 counts per min above background (cpm) under our standardized conditions of assay.

RESULTS

The amount of [^{14}C]-histamine injected in each experiment would give about 65,000 cpm under our conditions of assay. As may be seen in Fig. 1, in experiments A and B the arterial blood taken 0.5 to 2 min after the intraportal injection contained very little [^{14}C]-histamine (20 cpm or less per ml.). Already 2 min after the injection arterial blood contained some [^{14}C]-methylhistamine and a fair amount of [^{14}C]-methylimidazoleacetic acid. Thus the injected histamine disappeared very rapidly from the blood, where some of its metabolites appeared within 2 min. In the course

TABLE 1

THE DISTRIBUTION OF [^{14}C]-HISTAMINE AND SOME OF ITS METABOLITES IN THE BLOOD AND TISSUES OF TWO HEART-LUNG-LIVER PREPARATIONS

Samples taken 30 min after the injection of 32 μg [^{14}C]-histamine into the portal vein

Experiment A	Blood cpm	Heart (20 g) cpm	Lungs (10 g) cpm	Liver (86 g) cpm	Total cpm
Histamine	500	280	100	700	1,580
Methylhistamine	2,000	200	100	1,400	3,700
Methylimidazole-acetic acid	30,000	3,600	1,900	30,800	66,300
Imidazoleacetic acid	750	0	30	420	1,200
	33,250	4,080	2,130	33,320	72,780
Experiment B	**Blood cpm**	**Heart (15 g) cpm**	**Lungs (20 g) cpm**	**Liver (80 g) cpm**	**Total cpm**
Histamine	0	160	40	160	360
Methylhistamine	0	390	0	0	390
Methylimidazole-acetic acid	24,000	1,950	2,300	20,000	48,250
Imidazoleacetic acid	150	0	20	240	410
	24,150	2,500	2,360	20,400	49,410

of the experiment the concentration of labelled histamine and methylhistamine in the blood continued to fall and the concentration of labelled methylimidazoleacetic acid rose. There was never any measurable quantity of radioactive imidazoleacetic acid (free and conjugated) or acetylhistamine in the blood 30 min after the injection of [^{14}C]-histamine. The amounts of radioactive histamine, methylhistamine, methylimidazoleacetic acid and imidazoleacetic acid (free and conjugated) in the tissues at the end of the experiment are shown in Table 1. By far the greatest part of the ^{14}C was in the form of methylimidazoleacetic acid. [^{14}C]-Acetylhistamine was not present in measurable amounts.

In these heart-lung-liver preparations the injected histamine was very rapidly removed from the blood and metabolized. In order to see to what extent this was due to the liver, similar experiments were performed in heart-lung preparations, where the arterial blood was returned to the heart via a polythene catheter tied into the inferior vena cava well above the liver, which was thus excluded from the circulation. The results of 2 experiments in heart-lung preparations are shown in

TABLE 2

THE DISTRIBUTION OF [^{14}C]-HISTAMINE AND SOME OF ITS METABOLITES IN THE BLOOD AND TISSUES OF TWO HEART-LUNG PREPARATIONS

Readings made 30 min after the injection of 32 μg [^{14}C]-histamine into the inferior vena cava

Experiment C	Blood cpm	Heart (20 g) cpm	Lungs (21 g) cpm	Total cpm
Histamine	10,000	10,800	5,000	25,800
Methylhistamine	5,700	15,000	1,140	21,840
Methylimidazole-acetic acid	400	750	4,000	5,150
Imidazoleacetic acid	1,000	500	500	2,000
	17,100	27,050	10,640	54,790
Experiment D	**Blood cpm**	**Heart (15 g) cpm**	**Lungs (25 g) cpm**	**Total cpm**
Histamine	8,500	1,800	2,100	12,400
Methylhistamine	7,500	10,800	7,700	26,000
Methylimidazole-acetic acid	1,500	750	4,000	6,250
Imidazoleacetic acid	1,000	500	500	2,000
	18,500	13,850	14,300	46,650

the lower part of Fig. 1. It may be seen that the radioactive histamine disappeared much more slowly from the blood in these experiments. After 30 min there was still some unchanged histamine in the circulating blood. During the experiments the concentration of [^{14}C]-methylhistamine rose slowly. Very little [^{14}C]-methylimidazoleacetic acid appeared in the blood. Again there was little or no radioactive imidazoleacetic acid or acetylhistamine in the blood at the end of the experiment. The amounts of labelled histamine or histamine metabolites remaining in the tissues at the end of the experiments are given in Table 2. The blood and the tissues contained considerable quantities of unchanged histamine. Methylhistamine was the most important metabolite ; it occurred especially in the heart.

To find out if the methylation of histamine occurred in the blood, the heart or the lungs [^{14}C]-histamine was incubated with blood or heart or lung tissue and the

TABLE 3

METABOLISM OF [^{14}C]-HISTAMINE IN BLOOD, HEART AND LUNG TISSUE *IN VITRO*

[^{14}C]-Histamine was incubated with 2 g minced tissue or 2 ml. blood, suspended in 2 ml. phosphate buffer for 2 hr at 37° C

	Blood	Heart	Lung
Amount of [^{14}C]-histamine added (cpm)	375	375	375
Amount of [^{14}C]-methyl-histamine formed (cpm)	10	300	250

[^{14}C]-methylhistamine formed measured. The results of one such experiment are summarized in Table 3. It may be seen that both heart and lung tissue could methylate histamine *in vitro*. Under the same conditions there was little or no methylation in the blood sample.

DISCUSSION

In the present experiments it was found that injected histamine disappeared very rapidly from the circulating blood in heart-lung-liver preparations of cats. The amount of histamine injected corresponded to about 10 μg/kg. It was given intravenously in less than 10 sec, and yet arterial blood collected 0.5 to 2 min after the injection did not contain more than about 0.01 μg per ml. On the other hand, in the heart-lung preparations the injected histamine disappeared much more slowly from the blood. Steggerda, Essex & Mann (1935) found that heart-lung preparations of dogs did not inactivate measurable amounts of histamine when a dose of 20 to 50 mg was given. The rate of inactivation seen in our heart-lung preparations seems too slow to be of any physiological significance.

According to Brown, Tomchick & Axelrod (1959) the heart and the lungs of cats are as efficient as the liver in methylating histamine *in vitro*. The great difference in our experiments between heart-lung-liver preparations and heart-lung preparations as regards the metabolism of histamine is thus not likely to be due to differences in concentration of the methylating enzyme. To some extent the difference can be explained by the greater amount of tissue in the heart-lung-liver preparations. It should be noted, however, that in the heart-lung-liver preparations the main metabolite was methylimidazoleacetic acid, whereas in the heart-lung preparations it was methylhistamine. It is thus possible that in the absence of the liver the limiting factor was the lack of the enzyme responsible for the oxidation of methylhistamine.

It has been suggested that amine oxidase should be important for the inactivation of unchanged histamine *in vivo* (Zeller, Stern & Blanksma, 1956). In the present experiments, however, less than 4% of the injected histamine was converted to imidazoleacetic acid, the end product of the oxidation of histamine by amine oxidase or diamine oxidase (Zeller, 1951, and Zeller, Stern & Blanksma, 1956). Thus amine oxidase, which is present in the liver in great amounts (Blaschko, 1951), seems to play a minor role in the metabolism of histamine. On the other hand, the present experiments support earlier observations, according to which amine oxidase or a similar catalyst takes a part in the further oxidation of methylhistamine (see Lindell, Nilsson, Roos & Westling, 1960).

There was no evidence of acetylation of the injected histamine. This is in agreement with Livingston & Code (1955), who did not find any increase in the excretion of conjugated histamine in urine during an intraportal infusion of histamine in dogs.

REFERENCES

BEST, C. H. & MCHENRY, E. W. (1930). The inactivation of histamine. *J. Physiol.* (*Lond.*), **70**, 349–372.

BLASCHKO, H. (1951). Amine oxidase and amine metabolism. *Pharmacol. Rev.*, **4**, 415–458.

BROWN, D. D., TOMCHICK, R. & AXELROD, J. (1959). The distribution and properties of a histamine-methylating enzyme. *J. biol. Chem.*, **234**, 2948–2950.

DALE, H. H. & LAIDLAW, P. P. (1911). The physiological action of β-iminazolylethylamine. *J. Physiol.* (*Lond.*), **41**, 318–344.

GUGGENHEIM, M. & LOEFFLER, W. (1916). Das schicksal proteinogener amine im tierkörper. *Biochem. Z.*, **72**, 325–350.

KOBAYASHI, Y. & SCHAYER, R. W. (1956). *In vitro* demonstration of a new histamine-metabolizing enzyme. *Arch. Biochem.*, **63**, 480–481.

LINDAHL, K. M. (1958). On the biological methylation of histamine. *Acta chem. scand.*, **12**, 2050–2051.

LINDAHL, K. M. (1960). The histamine methylating enzyme system in liver. *Acta physiol. scand.*, **49**, 114–138.

LINDELL, S.-E., NILSSON, K., ROOS, B.-E. & WESTLING (1960). The effect of enzyme inhibitors on histamine. *Brit. J. Pharmacol.*, **15**, 351–355.

LINDELL, S.-E. & SCHAYER, R. W. (1958). The renal removal of injected [^{14}C]-histamine from the blood in dogs. *Brit. J. Pharmacol.*, **13**, 44–51.

LIVINGSTON, R. H. & CODE, C. F. (1955). Role of the liver in the conjugation of histamine in intact animals. *Amer. J. Physiol.*, **181**, 428–434.

ÖRSKOV, S. L. (1953). A liver-heart-lung preparation. *Acta physiol. scand.*, **27**, 328–332.

STEGGERDA, F. R., ESSEX, H. E. & MANN, F. C. (1935). The inactivation of histamine in perfused organs. *Amer. J. Physiol.*, **112**, 70–73.

ZELLER, E. A. (1942). Diamine oxidase. *Advanc. Enzymol.*, **2**, 93–112.

ZELLER, E. A. (1951). Diamine oxidase. *The Enzymes*, Vol. II, Part I, p. 544–558. Ed. SUMMER, J. B. & MYRBACK, K. N.Y. : Academic Press.

ZELLER, E. A., STERN, P. & BLANKSMA, L. A. (1956). Degradation of histamine by monoamine oxidase. *Naturwissenschaften*, **43**, 157.